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PRINCIPAL INVESTIGATOR: Qun Zhou, M.D., Ph.D.

CONTRACTING ORGANIZATION: Johns Hopkins University School of Medicine
Baltimore, Maryland 21205

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14. ABSTRACT The important role of polyamine in regulation of cell growth has led to the development of a number of polyamine analogues that can intervene in natural polyamine metabolism and inhibit the growth of tumor cells. This proposal was designed to elucidate the molecular mechanisms and the therapeutic efficacy of a new generation of polyamine analogues in treatment of human breast cancer. In the second year of this award, we demonstrated that activation of p53/p21 is important for the induction of polyamine analogue-induced growth inhibition and apoptosis. In our latest studies, we demonstrated that oligoamines specifically suppress the gene expression and function of the estrogen receptor α (ER α), a principal determinant of breast cell growth and differentiation, leading to the subsequent down-regulation of ER α -target genes. Simultaneous treatment with spermine could significantly reverse analogue-induced down-regulation of ER α expression and activity, suggesting that natural polyamines play an important role in maintaining normal ER α activity. We also demonstrated that oligoamines inhibit the ER α promoter element, which contains the GC and C/A rich boxes bound by Sp1 transcription factor family. Moreover, ER α has been observed to be an important mediator of the effects of the transcription factor NF- κ B in response to analogue. These preliminary data indicate the possibility of a novel regulation of estrogen signaling by polyamine analogues.					
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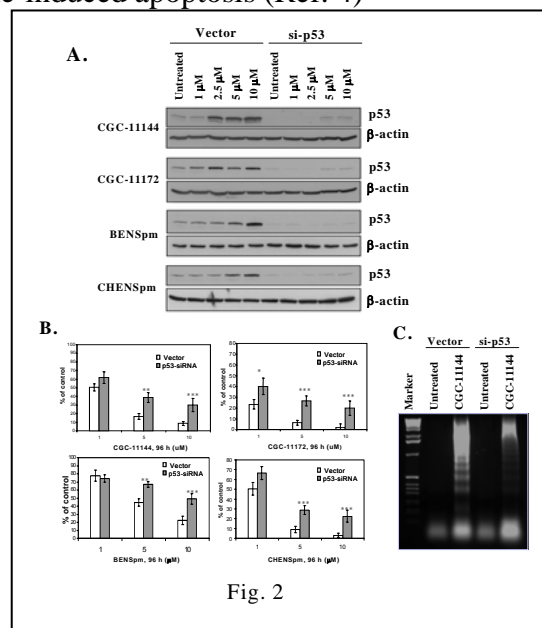
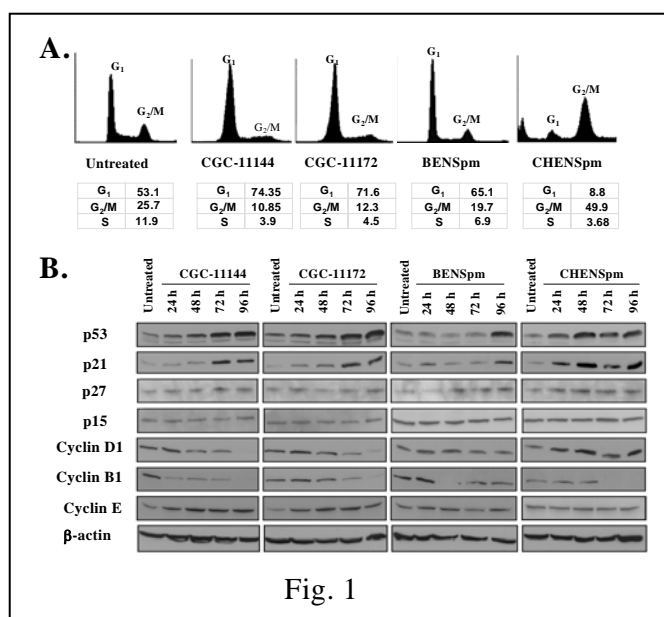
Introduction

Breast cancer is the most common malignancy and a leading cause of mortality in women in the Western world. More than 200,000 American women will be diagnosed with breast each year (Ref. 1). The natural polyamines (putrescine, spermidine, and spermine) are essential for cell growth and proliferation. A number of polyamine analogues have been developed to intervene in natural polyamine metabolism and inhibit the growth of tumor cells. Synthetic polyamine analogues can mimic the natural polyamines in their self-regulatory role and down-regulate polyamine biosynthesis by feedback mechanisms, but they are unable to act as substitutes for natural polyamines in cell growth. The precise mechanisms of antitumor activities of polyamine analogues are not entirely understood (Ref. 2). Recently, a new generation of polyamine analogues termed oligoamines with longer chains than natural polyamine molecules has been developed. Preliminary results showed that these novel polyamine analogues were effective against human breast cancer cells. The purpose of this project is to elucidate the molecular mechanisms and the therapeutic efficacy of a novel class of polyamine analogues in the treatment of human breast cancer. This will be accomplished by the studies of three related technical objectives. They include: 1) to investigate the *in vitro* molecular mechanisms responsible for the growth inhibition and apoptosis induced by novel polyamine analogues in breast cancer cells, 2) to expand the *in vitro* studies to an *in vivo* animal model of human breast cancer, 3) to study the therapeutic efficacy of combinations of novel promising polyamine analogues with clinically active antineoplastic agents.

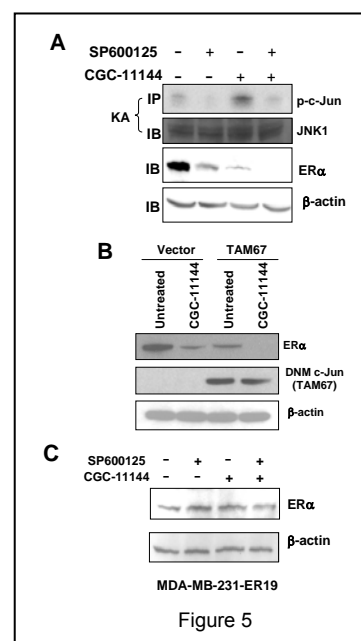
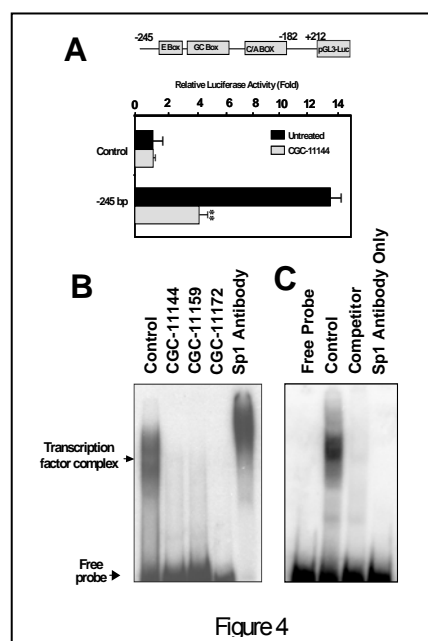
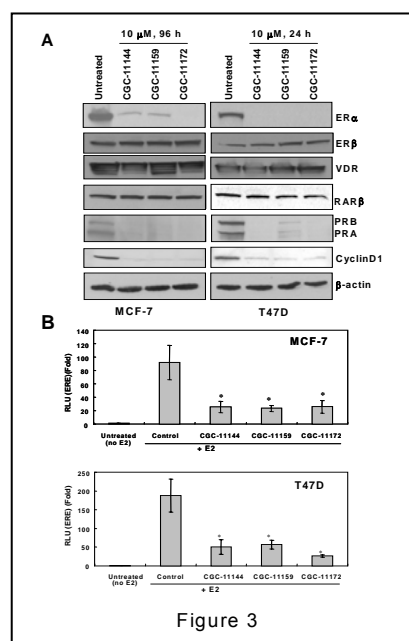
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Technical Objective 1: To investigate *in vitro* molecular mechanisms responsible for the growth inhibition and apoptosis induced by novel polyamine analogues in breast cancer cells.

In the first year of the award, we have demonstrated that a novel analogue, SL-11144, has effective antineoplastic action against human breast cancer cells *in vitro* and *in vivo*, and multiple apoptotic mechanisms are associated with its cytotoxic effect in specific human breast cancer cell lines (Ref. 3). We also demonstrated that Jun NH₂-terminal kinase (JNK)/AP-1 signaling pathway plays a protective role in oligoamine-induced apoptosis (Ref. 4)



We further demonstrated that specific polyamine analogues exhibit differential inhibitory effects against the growth of human breast cancer MCF-7 cells. Treatment of MCF-7 cells with two oligoamine analogues and the symmetrically substituted bis(alkyl)-substituted analogue, BENSpm, produced a G₁ cell cycle arrest, while the unsymmetrically substituted bis(alkyl)-substituted analogue, CHENSpm, induced a G₂/M cell cycle arrest. All four compounds significantly up-regulated p53 and p21 expression in MCF-7 cells (Fig. 1, Ref. 5). Stable transfection of small interfering RNA (siRNA) targeting p53 blocked the expression of p21 induced by the polyamine analogues and significantly reduced polyamine analogue-induced growth inhibition and apoptosis, suggesting that polyamine analogue-induced p21 expression occurs through p53-dependent mechanisms. The effects of analogue exposure on cyclins and cyclin dependent kinases varied with the specific agent used (Fig. 2, Ref. 5). In our latest studies, we demonstrated that oligoamines specifically suppress the gene expression and the ligand-dependent transcriptional activity of the estrogen receptor α (ER α), a principal determinant of breast cell growth and differentiation, leading to the subsequent down-regulation of ER α -target genes such as progesterone receptor (PR) and cyclinD1 (Fig. 3, Ref. 6). We also



demonstrated that oligoamines inhibit the ER α promoter element, which contains the GC and C/A rich boxes bound by Sp1 transcription factor family (Fig. 4, Ref. 6). Moreover, treatment of MDA-MB-231 cells with the JNK specific inhibitor SP600125, or expression of the c-Jun dominant negative inhibitor, TAM67, blocked the oligoamine-activated JNK/c-Jun pathway and enhanced oligoamine-inhibited ER α expression, suggesting that AP-1 is a positive regulator of ER α expression and that oligoamine activated JNK/AP-1 activity may antagonize the down-regulation of ER α induced by oligoamines (Fig. 5, Ref. 6). Taken together, these results suggest a novel antiestrogenic mechanism for specific polyamine analogues in human breast cancer cells.

Technical Objective 2: To expand the *in vitro* studies to an *in vivo* animal model of human breast cancer.

Based on the *in vitro* data, the selection and evaluation of the promising novel polyamine analogues for further *in vivo* animal study is underway. The *in vivo* studies are expected to begin late this year.

Technical Objective 3: To study the therapeutic efficacy of combinations of novel polyamine analogues with clinically active antineoplastic agents.

Performance of this technical objective will be initiated after the completion of the *in vivo* animal study. Results from Technical Objective 2 are needed to guide the design of the combination studies. This part of designed studies is expected to start early next year.

Key Research Accomplishments

- In MCF-7 breast cancer cells, upregulation of p21^{WAF1/CIP1} by polyamine analogues is p53 dependent and analogue-structure dependent.
- Oligoamines significantly down-regulate Estrogen Receptor alpha (ER α), which may contribute to polyamine analogue-induced apoptosis in human breast cancer cells.

Reportable Outcomes (supported by this grant since June 2004)

Peer-reviewed research articles, review and chapter

1. **Huang, Y.**, Pledge A.M., Casero, R.A., Davidson, N.E. Molecular mechanisms of polyamine analogues in cancer cells. *Anti-Cancer Drugs*, 16(3): 229-241, 2005.
2. **Huang, Y.**, Pledge A., Rubin E., Marton, L.J., Woster, P.M., Sukumar, S., Casero, R.A., and Davidson, N.E. Role of p53/p21^{WAF1/CIP1} activation in the mediation of polyamine analogue induced growth inhibition and cell death in human breast cancer cells. *Cancer Biol. Ther.*, 4(9):1006-1013, 2005.
3. Pledge A.M., **Huang, Y.**, Hacker, A., Zhang Z., Woster, P.M., Davidson, N.E., and Casero, R.A.. Spermine oxidase SMO(PAOh1), not N¹-acetylpolyamine oxidase (PAO) is the primary source of cytotoxic H₂O₂ in polyamine analogue-treated human breast cancer cell lines. *J. Biol. Chem.*, 280(48): 39843-39851, 2005.
4. **Huang, Y.**, and Davidson, N.E. Book Chapter: Breast Cancer. In: *Principles of Molecular Medicine* (2nd ed). Runge, M., and Patterson, WC. (eds), Humana Press, 2006.
5. **Huang, Y.**, Keen, J.C., Pledge A., Marton, L.J., Zhu, T., Sukumar, S., Park, B.H., Blair, B.G., Brenner, K., Casero, R.A., and Davidson, N.E. Polyamine Analogues Down-regulate Estrogen Receptor α Expression in Human Breast Cancer Cells. *J. Biol. Chem.*, 281(28): 19055-19063, 2006.

Conference Abstracts

1. **Huang, Y.**, et al. Estrogenic regulation of polyamine analogues in human breast cancer cells. Ninety sixth Annual Meeting of American Association of Cancer Research, Los Angeles, 2005, Proceedings of the American Association for Cancer Research, 46: 547.
2. **Huang, Y.**, et al. Distinct roles of p53/p21^{WAF1/CIP1} and JNK/Jun activation in the mediation of polyamine analogue induced growth inhibition and cell death in human breast cancer MCF-7 cells (Poster). Ninety sixth Annual Meeting of American Association of Cancer Research, Los Angeles, 2005, Proceedings of the American Association for Cancer Research, 46: 535.
3. **Huang, Y.**, et al. Estrogenic regulation of polyamine analogues in human breast cancer cells. The fourth Era of Hope meeting for the Department of Defense (DOD) Breast Cancer Research Program (BCRP), Philadelphia, PA, 2005.
4. Pledge, A.M., **Huang, Y.**, et al. Differential induction of human spermine oxidase (PAOh1/SMO) mRNA and activity by a polyamine analogue in human breast cancer cell lines. The fourth Era of Hope meeting for the Department of Defense (DOD) Breast Cancer Research Program (BCRP), Philadelphia, PA, 2005.

Conclusions

A series of studies have been carried out to evaluate the *in vitro* molecular mechanisms responsible for the growth inhibition and apoptosis induced by novel polyamine analogues in human breast cancer cells. Our initial investigations have identified a novel class of polyamine analogues termed oligoamines as promising antitumor agents in treatment of human breast cancer. Further studies revealed the involvement of p53/p21 and JNK/AP1 signaling pathways in polyamine analogue-induced growth inhibition and apoptosis. We also demonstrated that oligoamines specifically suppress the expression and ligand-independent transcriptional activity of the ER α through inhibition of the binding of the transcription factor, Sp1, to ER α promoter region. Decreased ER α results in the subsequent down-regulation of ER α -target genes like PR and cyclinD1, which may contribute to cell growth inhibition. Examination of the molecular mechanisms of polyamine analogues in human breast cancer cells will be continued as scheduled in objectives 2 and 3.

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1. **Huang, Y.**, and Davidson, N.E. Book Chapter: Breast Cancer. In: *Principles of Molecular Medicine* (2nd ed). Runge, M., and Patterson, WC. (eds), Humana Press, 2006.
2. **Huang, Y.**, Pledge A.M., Casero, R.A., Davidson, N.E. Molecular mechanisms of polyamine analogues in cancer cells. *Anti-Cancer Drugs*, 16(3): 229-241, 2005.
3. **Huang, Y.**, Hager, E.R., Phillips, D.L., Dunn, V.R., Hacker, A., Frydman, B., Kink J.A., Valasinas, A.L., Reddy, V.K., Marton, L.J., Casero, R.A., and Davidson, N.E. A Novel Polyamine Analog Inhibits Growth and Induces Apoptosis in Human Breast Cancer Cells. *Clin. Cancer. Res.*, 9: 2769-2777, 2003.
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6. **Huang, Y.**, Keen, J.C., Pledge A., Marton, L.J., Zhu, T., Sukumar, S., Park, B.H., Blair, B.G., Brenner, K., Casero, R.A., and Davidson, N.E. Polyamine Analogues Down-regulate Estrogen Receptor α Expression in Human Breast Cancer Cells. *J. Biol. Chem.*, 281(28):19055-63, 2006.

Appendices

1. Copy of article published in *Cancer Biol. & Ther.*, 4(9):1006-1013, 2005.
2. Copy of article published in *J. Biol. Chem.*, 280(48): 39843-39851, 2005.
3. Copy of article published in *J. Biol. Chem.*, 281(28): 19055-19063, 2006.

Research Paper

Role of p53/p21^{Waf1/Cip1} in the Regulation of Polyamine Analogue-Induced Growth Inhibition and Cell Death in Human Breast Cancer Cells

Yi Huang¹

Allison Pledgie¹

Ethel Rubin¹

Laurence J. Marton²

Patrick M. Woster³

Saraswati Sukumar¹

Robert A. Casero, Jr.¹

Nancy E. Davidson^{1,*}

¹The Sidney Kimmel Comprehensive Cancer Center; Johns Hopkins University School of Medicine; Baltimore, Maryland USA

²CellGate Inc.; Redwood City, California USA

³Department of Pharmaceutical Sciences; College of Pharmacy and Allied Health Professions; Wayne State University; Detroit, Michigan USA

*Correspondence to: Nancy E. Davidson; Breast Cancer Program; The Sidney Kimmel Comprehensive Cancer Center; Johns Hopkins University School of Medicine; 1650 Orleans Street, Room 409; Baltimore, Maryland 21231 USA; Tel.: 410.955.8489; Fax: 410.614.4073; Email: davidna@jhmi.edu

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KEY WORDS

polyamine analogues, breast cancer cells, cell cycle arrest, cell death, p53/p21^{Waf1/Cip1}

ABBREVIATIONS

ODC	ornithine decarboxylase
SAMDC	S-Adenosylmethionine decarboxylase
PAO	polyamine oxidase
SMO/PAOh1	Spermine Oxidase
DFMO	α -difluoromethylornithine
BENSpm	N ¹ , N ¹¹ -bis(ethyl)nonospermine
CHEMSpm	N ¹ -(cycloheptylmethyl)-N ¹¹ -ethyl-4,8-diazaundecane
MTT	3-(4,5-dimethyl-2-yl)-2,5-diphenyl-tetrazolium
DMSO	Dimethyl Sulfoxide
PBS	phosphate-buffered saline
PAGE	polyacrylamide gel electrophoresis
SSAT	spermidine/spermine N ¹ -acetyltransferase

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ABSTRACT

Intracellular polyamines are absolutely required for cell proliferation and many tumors have abnormal requirements for polyamines. Therefore, the polyamine metabolic pathway represents a rational target for antineoplastic intervention. A number of polyamine analogues act as potent modulators of cellular polyamine metabolism and exhibit encouraging effects against tumor growth in both cell culture and animal studies. In this study we demonstrate that specific polyamine analogues exhibit differential inhibitory action against growth of human breast cancer MCF-7 cells. Treatment of MCF-7 cells with oligoamine analogues and the symmetrically substituted bis(alkyl)-substituted analogue, BENSpm, produced a G₁ cell cycle arrest, while the unsymmetrically substituted bis(alkyl)-substituted analogue, CHENSpm, induced a G₂/M cell cycle arrest. All four compounds significantly upregulated p53 and p21 expression in MCF-7 cells. Stable transfection of small interfering RNA (siRNA) targeting p53 blocked the expression of p21 induced by the polyamine analogues and significantly reduced polyamine analogue-induced growth inhibition and apoptosis, suggesting that polyamine analogue-induced p21 expression occurs through p53-dependent mechanisms. The effects of analogue exposure on cyclins and cyclin dependent kinases varied with the specific agent used. Expression of p53 siRNA reversed only BENSpm-modulated the cell cycle arrest, suggesting that regulation of cell cycle arrest by p53/p21 induced by polyamine analogues occurs through agent-specific mechanisms. Understanding the mechanism of p53-mediated cellular responses to polyamine analogue may help to improve the therapeutic efficacy of polyamine analogues in human breast cancer.

INTRODUCTION

Polyamines are naturally occurring polycations that are required for cell growth, and manipulation of cellular polyamine levels can lead to decreased proliferation, and, in some cases, increased cell death. Natural polyamine biosynthesis is regulated by the rate-limiting enzymes ornithine decarboxylase (ODC) and S-Adenosylmethionine decarboxylase (SAMDC), while polyamine catabolism is driven by spermidine/spermine N¹-acetyltransferase/polyamine oxidase (SSAT/PAO) and spermine oxidase SMO(PAOh1).¹⁻³ The requirement for polyamine action in cell proliferation and tumorigenesis has underscored the rationale of targeting polyamine metabolism as a therapeutic strategy.^{4,5} Polyamine analogues have been synthesized as metabolic modulators that deplete natural intracellular polyamine pools, or polyamine mimetics that displace the natural polyamines from binding sites, but do not substitute for their growth promoting function.^{2,6} Symmetrically substituted bis(alkyl)polyamine analogues represent the first generation of these analogues, some of which downregulate polyamine biosynthesis and increase SSAT activity in certain tumor cell types like non-small cell lung cancer cells, melanoma and human breast cancer cells.⁷⁻⁹ A second generation of polyamine analogues are unsymmetrically substituted compounds that display structure-dependent and cell type-specific effects on regulation of polyamine metabolism.¹⁰ Recently, a series of new polyamine analogues designated conformationally restricted, cyclic and oligoamine analogues have been developed.¹¹⁻¹³ Some of these agents incorporate alterations that limit the free rotation of the single bonds in otherwise flexible molecules such as spermine or its analogues, thus restricting the molecular conformation that they may assume. Oligoamine analogues consist of synthetic octa-, deca-, dodeca- and tetradecamines with longer chains than natural mammalian polyamine molecules, with or without conformational restriction. Some of these novel analogues have shown significant activity against multiple human tumors both in vitro and in vivo.^{6,12,13}

Although it is clear that polyamine analogues can induce cell death, the mechanisms remain elusive. Interaction with DNA, displacement of natural polyamines from their

Figure 1. Structures of natural polyamines and polyamine analogues used in this study.

binding sites, induction of polyamine catabolic enzyme activity and depletion of mitochondrial DNA have all been proposed as possible mechanisms underlying the anti-tumor action of polyamine analogues. Polyamines are required at different stages of cell cycle progression, and use of polyamine biosynthesis inhibitors or analogues to target the polyamine biosynthetic and catabolic machinery can disrupt normal cell cycle regulation and subsequently lead to the cessation of tumor cell growth. For example, treatment of human breast cancer MCF-7 cells with the specific ODC inhibitor, DFMO, led to an increased level of cyclin B1 in early G₁ phase.¹⁴ A symmetrically substituted analogue, BENSpm, was demonstrated to upregulate the expression of the tumor suppressor, p53, and the CDK inhibitor, p21, in a human melanoma cell line.¹⁵ Recent studies indicate that apoptotic cell death might be a common characteristic for polyamine analogue-induced cytotoxicity in cancer cells. Although the 'super-induction' of SSAT and H₂O₂ produced by polyamine catabolism has been implicated in the cytotoxic response of specific solid tumor phenotypes to specific analogues such as BENSpm and CPENSpm,¹⁵⁻¹⁷ other analogues like oligoamines do not highly induce polyamine catabolic enzymes but can still inhibit tumor cell growth and induce apoptosis.¹⁸ We have recently demonstrated that multiple apoptotic mechanisms were associated with the oligoamine CGC-11144-induced cytotoxic effects in specific breast cancer cell lines.¹⁹

These findings indicate that polyamine analogue-induced cell growth inhibition and death may occur through multiple structure-specific and cell type-specific mechanisms.

To elucidate the molecular mechanisms of polyamine analogue cytotoxicity as well as the structural determinants for cytotoxicity in human breast cancer cells, several well-characterized polyamine analogues with different structures were analyzed for their effects on cell cycle and apoptotic regulatory pathways in MCF-7 cells. Our findings indicate that the oligoamine analogues (CGC-11144 and CGC-11172) and the symmetrically substituted alkylpolyamine analogue, BENSpm, arrested MCF-7 cells at G₁ phase, while the unsymmetrically substituted alkylpolyamine analogue, CHENSpm, led to G₂/M arrest. All four analogues significantly upregulated p53/p21 levels in MCF-7 cells, and suppression of p53 activation by siRNA abrogated p21 activation by polyamine

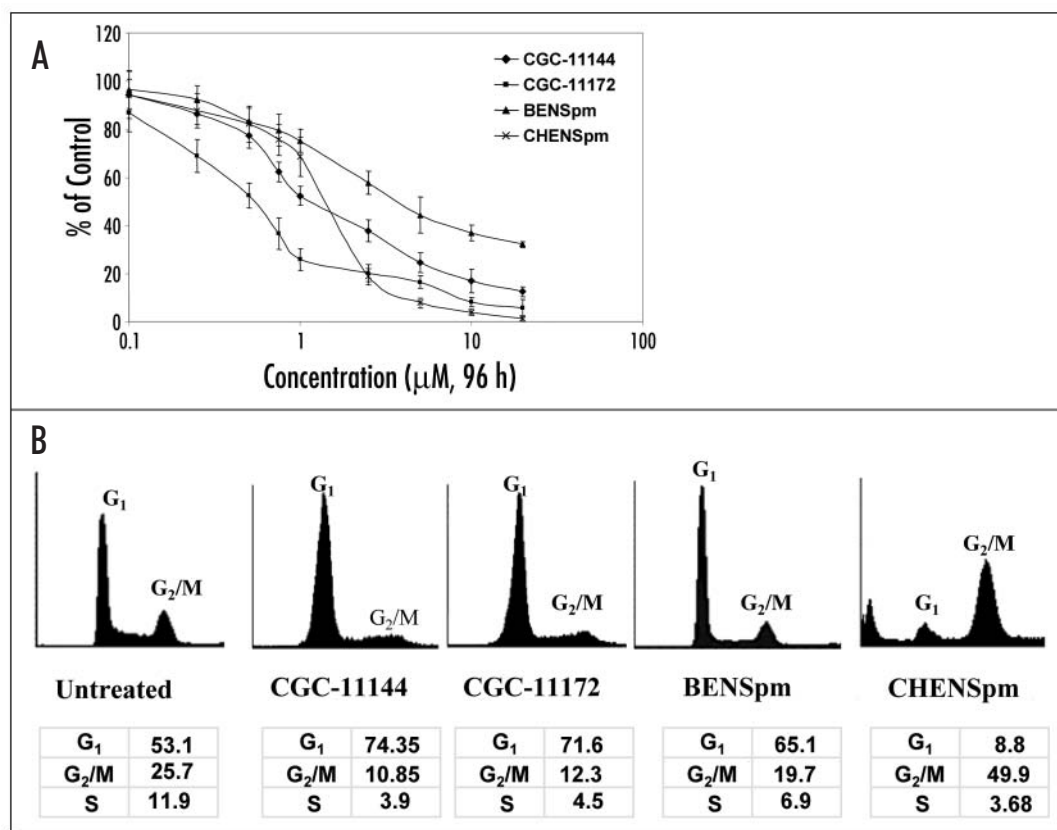
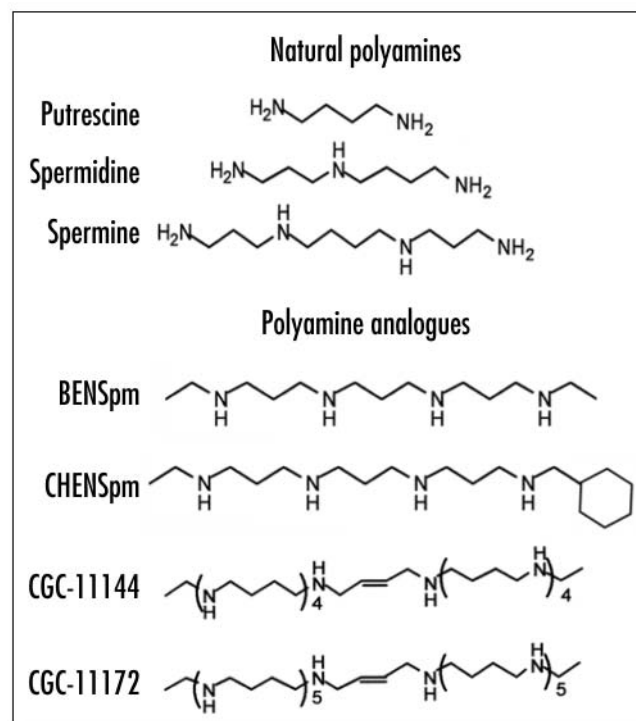


Figure 2. Polyamine analogues inhibit growth and induce cell cycle arrest in MCF-7 cells. (A) Cells were treated with increasing concentrations of the indicated polyamine analogues and analyzed by MTT assays. Shown are means \pm S.D. of three independent experiments performed in quadruplicate. (B) Cells were incubated with 10 μ M of polyamine analogue for 72 h, harvested, and stained for DNA with Hoechst 33258 for flow cytometry. The peaks corresponding to G₁ and G₂/M phases of the cell cycle are indicated. Each experiment was performed twice with similar results.

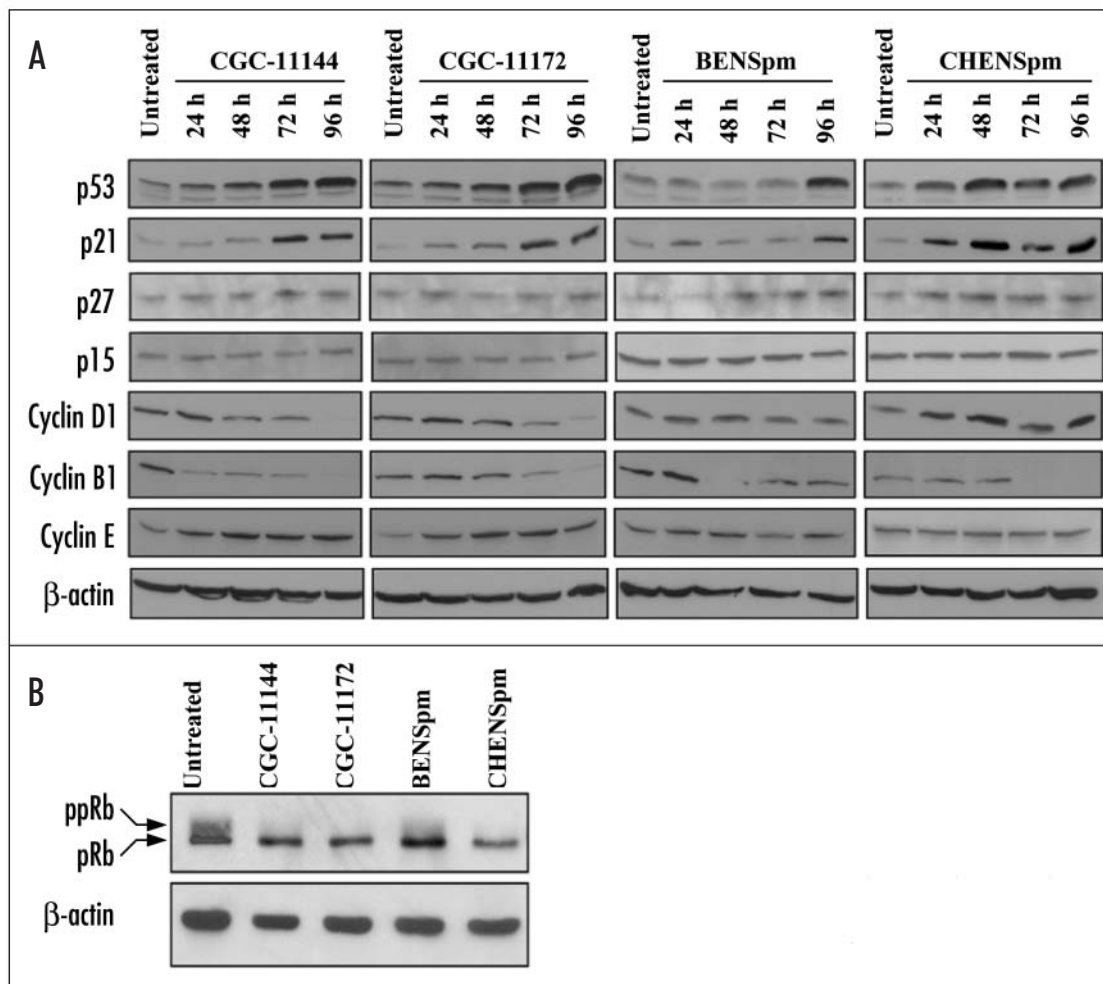


Figure 3. Effects of polyamine analogues on cell cycle regulatory proteins. (A) MCF-7 cells were treated with 10 μ M polyamine analogue for 24-96 h (A) or 96 h (B). Equal amounts of protein (50 μ g/lane) were fractionated on 12% (A) or 6% (B) SDS-PAGE gels (6% gel for pRb) and transferred to PVDF or nitrocellulose membranes followed by immunoblotting with different monoclonal or polyclonal antibodies. Actin protein was blotted as a control. Each experiment was performed twice with similar results.

analogues and significantly reduced polyamine analogue-induced growth inhibition and apoptosis. However, inhibition of p53/p21 activation reversed only BENSpM induced cell cycle arrest, suggesting that effects of p53/p21 on polyamine analogue-induced cell cycle arrest occur through agent-specific mechanisms.

METHODS

Compounds and culture conditions. N^1 , N^{11} -bis(ethyl)norspermine (BENSpM), N^1 -ethyl- N^{11} -(cycloheptyl)methyl-4,8,diazaundecane (CHENSpM), CGC-11144 and CGC-11172 (previously named SL-11144 and SL-11172) were synthesized as previously reported.^{11,13,20} Stock solutions (10 mM in ddH₂O) of each analogue were diluted with medium to the desired concentrations for specific experiments. The human breast cancer MCF-7 cells were maintained in DMEM supplemented with 5% fetal bovine serum, 2 mM glutamine and incubated at 37°C in a 5% CO₂ atmosphere.

MTT growth inhibition assays. Growth inhibition was assessed by MTT assays as described previously.¹⁸ Briefly, 2,000–5,000 cells were plated in 96-well dishes and treated with increasing concentrations of compounds for the indicated times. All of the experiments were plated in quadruplicate and were carried out at least three times. The results of assays are presented as means \pm S.D. of all determinants.

Western blotting. After treatment, cells were harvested, and proteins (50 μ g/lane) were fractionated on 12% SDS-PAGE gels, transferred to PVDF membranes and immunoblotted with antibodies as indicated. Primary antibodies against p53, p21, p27, cyclin D1, cyclin B1, CDK2, CDK4 and CDK6 were purchased from Santa Cruz Biotechnology Company (Santa Cruz, CA). Antibodies against p15 and cyclin E1 were from Oncogene Science (Cambridge, MA). To detect pRb protein, one hundred micrograms of total protein per sample was separated by 6% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with monoclonal antibody RB-PMG3-245 (Pharmingen, UK) as described previously.²¹ Actin was used to normalize for protein loading. All experiments were performed at least twice with similar results.

Plasmid construction and stable transfection. Small interfering RNA (siRNA) oligonucleotides targeting p53 (sense, 5'-GACTCCAG-TGGTAATCTAC-3'; anti-sense, 5'-GTAGATTACCAC-TGGAGTC-3') were designed and synthesized based on previously published sequences.²² The complementary oligonucleotides

were ligated into pSilencer siRNA expression vectors (Ambion, Austin, Texas). Transfections were performed with LipofectAMINE PLUSTM reagent (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. Stable transfectants were selected by incubating the cells in medium containing 500 μ g/ml geneticin (G418). Cells from individual colonies were examined for p53 expression by Western blot.

Determination of internucleosomal DNA cleavage. After treatment, cells were harvested, counted, and washed with phosphate-buffered saline (PBS) at 4°C. Cells were then suspended in lysis buffer (5 mM Tris-HCl, 20 mM EDTA and 0.5% Triton X-100) and incubated for 20 min on ice. Detection of DNA fragmentation was performed as described previously.¹⁸ DNA samples were analyzed by electrophoresis in a 1.2% agarose slab gel containing 0.2 mg/ml ethidium bromide, and visualized under UV illumination. The experiments were performed at least twice with similar results.

Immunoprecipitation and kinase assays. The immunoprecipitation and kinase assays were performed as described previously.²³ Cells were harvested by trypsinization and washed with PBS buffer. The cell pellet was resuspended in 1 ml of immunoprecipitation lysis buffer (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 1% Nonidet P-40, 50 mM EDTA, 1x protease inhibitor) and was rotated in a rotary shaker for 30 min at 4°C. Kinase complexes were immunoprecipitated by incubation for 2h at 4°C with the indicated rabbit polyclonal or monoclonal antibodies (Santa Cruz Biotechnology) bound to

protein-A or G Sepharose (Amersham Biosciences). The immunoprecipitates were washed three times with lysis buffer and twice with kinase buffer (50 mM HEPES, pH 7.4, 10 mM-glycerophosphate, 10 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate). The kinase assays were initiated by the addition of 2 µg of substrate protein, 20 µM cold ATP and 10 µCi [γ -³²P] ATP. Reaction mixtures were incubated for 30 min at 30°C and stopped by the addition of 2xSDS-PAGE sample buffer. The phosphorylation of the substrate proteins was analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. The experiments were performed at least twice with similar results.

Flow cytometry analysis. Cells were harvested, counted, washed with phosphate-buffered saline (PBS) at 4°C, fixed with formaldehyde, and stained with Hoechst 33258(Sigma). BD-LSR was used to perform FACS and the cell cycle was analyzed using Cell-Quest software.

Statistical analysis. The Student's t test was used to determine the statistical differences between various experimental and control groups. $p \leq 0.05$ was considered significant.

RESULTS

Growth inhibition and cell cycle arrest by polyamine analogues. BENSpm is a symmetrical bisalkylated analogue with structural similarity to natural polyamines, while CHENSpm, an unsymmetrically substituted analogue, is structurally different from BENSpm with one terminal ethyl group being substituted by a cycloheptylmethyl group. The oligoamines, CGC-11144 and CGC-11172, are alkylamines with longer chains than natural polyamines and each NH₂⁺ residue is separated by four CH₂ residues with central cis double bonds that restrict the analogue conformation (Fig. 1). The sensitivity of estrogen receptor α (ER) positive, p53 wild type human breast cancer MCF-7 cells to these polyamine analogues was assessed by MTT growth inhibition assay. The IC₅₀ values for oligoamines are about 0.75–1.5 µM for 96 h treatment. The bisalkylated analogue, CHENSpm, exhibited a lower IC₅₀ value (~2.5 µM) than that of another bisalkylated analogue BENSpm (~5–7.5 µM) (Fig. 2A). Flow cytometric analysis indicated an increase in the proportion of cells in the G₁ phase of the cell cycle after treatment with CGC-11144, CGC-11172 or BENSpm for 72 h, whereas CHENSpm arrested MCF-7 cells at the G₂/M phase (Fig. 2B). These results suggest that modifications in analogue structure may affect the drug cytotoxicity and cell cycle regulation in MCF-7 cells.

Effects of polyamine analogues on cell cycle regulatory proteins. A number of cell cycle regulatory genes have been demonstrated to play important roles in controlling cell growth and death. These genes include the tumor suppressors p53 and pRb, cyclins, CDKs, CDK inhibitors (CDKi) of the Cip/Kip family p21^{Waf1/Cip1} and p27, and the INK4 family of CDKi, p15 and p16.²⁴ To determine whether these proteins are involved in the mediation of polyamine analogue-induced cell cycle arrest and death in human breast cancer cells, their expression in MCF-7 cells after treatment with 10 µM polyamine analogue for 24 h to 96 h was examined. All analogues studied induced p53 and p21 protein expression, suggesting that p53/p21 pathway activation is a common cellular response of MCF-7 cells to different polyamine analogue treatment (Fig. 3A). The expression of another member of the Cip/Kip family, p27^{kip1}, was unaffected by each of the analogue. No analogue altered the p15 protein levels and p16 expression was essentially undetectable in MCF-7 cells under the conditions examined (data not shown). Cyclin D1, which is complexed with either CDK4 or CDK6, controls cell cycle progression in the G₁ phase by enabling the enzymes to phosphorylate retinoblastoma protein (pRb). Protein levels of cyclin D1 were downregulated by oligoamines after 72 h treatment, but were virtually unchanged by BENSpm and somewhat increased by CHENSpm. Cyclin B1 interacts with Cdk1 (CDC2) to form an active complex that drives cellular progression from G₂- to M-phase. All four analogues downregulated cyclin B1 but at different time points. Cyclin E forms a complex with CDK2 to target components of the DNA synthesis machinery, leading to the assembly of complexes necessary for DNA replication at the G₁/S-phase. Oligoamines but not bis(alkyl)-substituted analogues increased cyclin E expression.

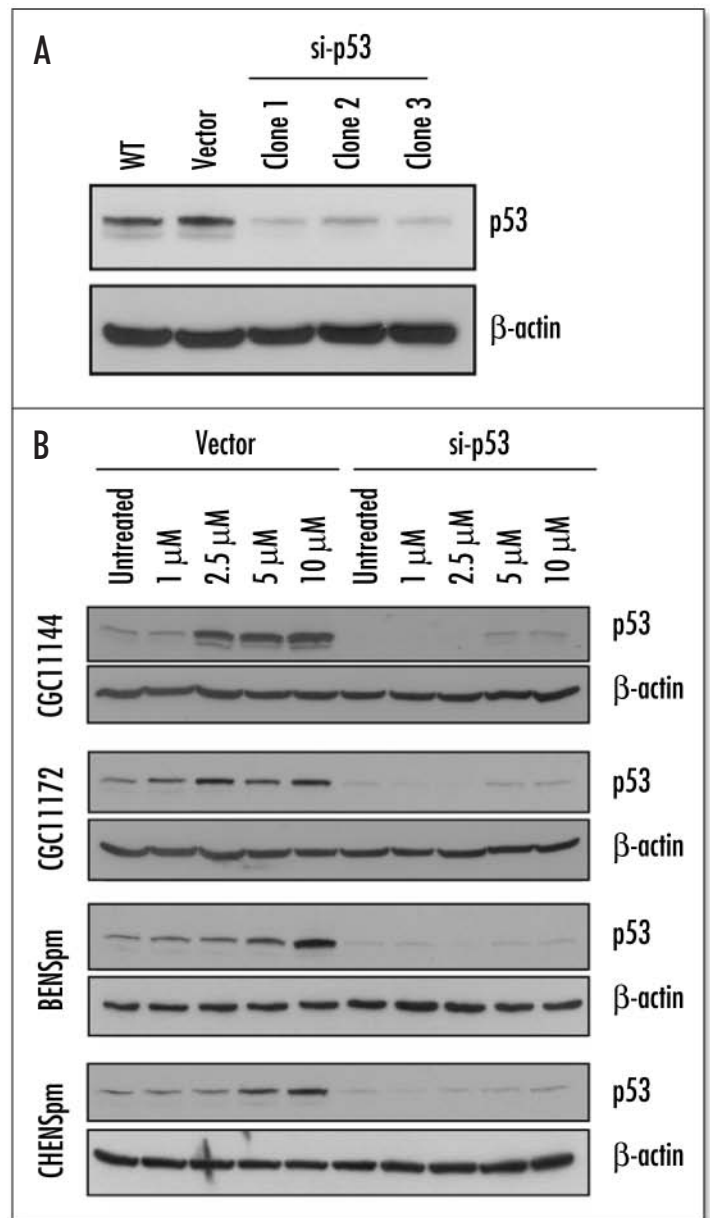


Figure 4. Suppression of p53 by p53 siRNA. (A) MCF-7 cells were stably transfected with empty pSilencer or pSilencer-p53 siRNA vector. Proteins isolated from parental, empty vector transfectants and p53-siRNA transfected single colonies were subjected to immunoblotting with an antibody to p53. Three geneticin-resistant clones were shown to have suppressed p53 level by si RNA. (B) Vector and p53 siRNA transfected MCF-7 cells were treated with 1–10 µM polyamine analogue for 96 h and subjected to immunoblotting with an antibody to p53.

pRb functions as a master switch of the cell cycle and tumor suppressor whose hyperphosphorylation can be prevented by CDK inhibitors (p21, p27, etc) and blocks cell cycle progression by interacting with transcription factors such as E2F. Interestingly, complete dephosphorylation of pRb was observed in MCF-7 treated by oligoamines and CHENSpm, while BENSpm induced the dephosphorylated form of pRb (lower band) and reduced the hyperphosphorylated form (upper band) (Fig. 3B). These results indicated that maintenance of pRb in the hypophosphorylated state is a common growth inhibitory mechanism shared by each of the analogues.

Suppression of p53 expression by p53 siRNA. To study the specific role of p53 activation in polyamine analogue induced cell cycle arrest and growth

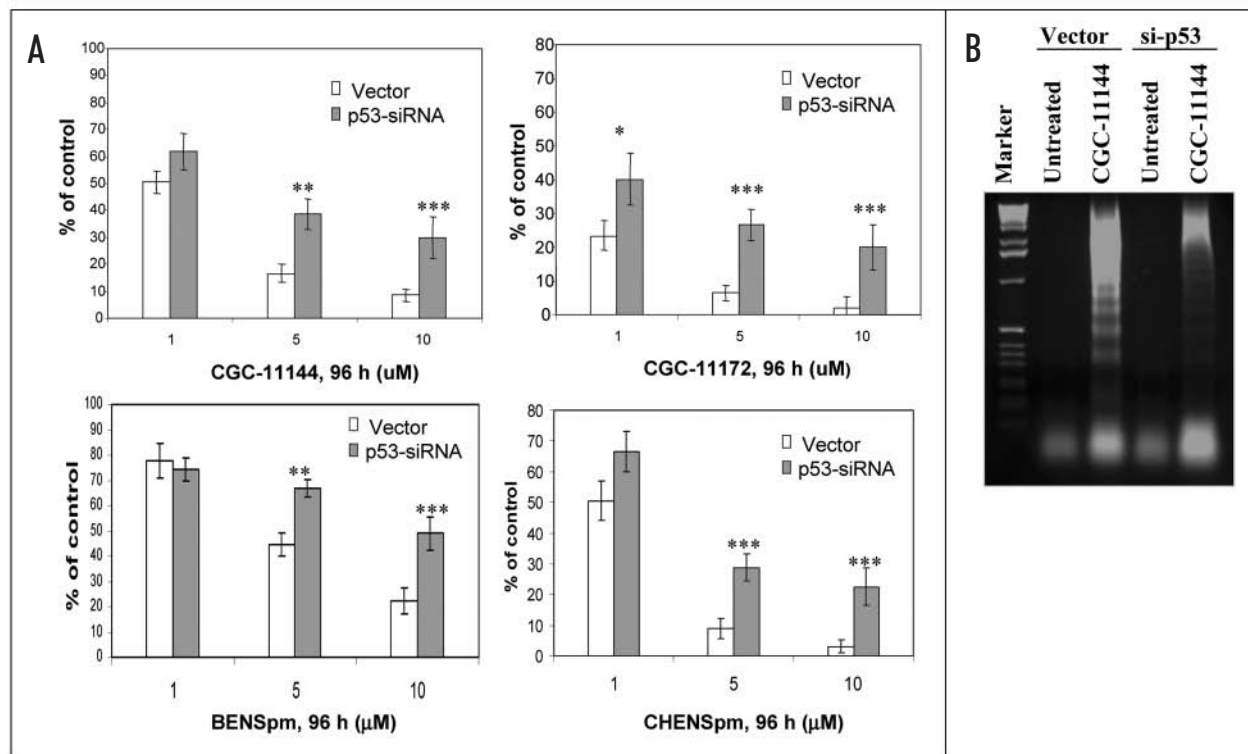


Figure 5. Effects of p53 siRNA on polyamine analogue-induced growth inhibition and apoptosis. (A). Vector and p53 siRNA transfected MCF-7 cells were treated with 1–10 μ M polyamine analogue for 96 h and analyzed by MTT assays. Shown are means \pm S.D. of three independent experiments performed in quadruplicate. There were statistically significant differences between transfected cells and vector transfected cells treated by $>5 \mu$ M CGC-11144, BENSpM, CHENSpM and $>1 \mu$ M CGC-11172. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student's *t*-test). (B) Vector and p53 siRNA transfected MCF-7 cells were treated with 10 μ M CGC-11144 for 96 h. Fragmented DNA was analyzed by electrophoresis in 1.2% agarose gel containing 0.1% ethidium bromide. Lane 1 is 1-kilobase DNA markers. The experiment was performed twice with similar results.

inhibition, siRNA was used to knock down the expression of p53 in MCF-7 cells. Stable transfection of a p53 pSilencer-vector suppressed endogenous p53 expression by approximately 80–90% in three clones (Fig. 4A), while the empty vector transfection showed no change in p53 expression. These three colonies were selected for the next series of experiments. Analytic data from all these three pairs of transfectants showed similar results. Here, analysis of clone #3 is presented. The vector control and p53 siRNA transfectants were treated with increasing concentrations (1–10 μ M) of each polyamine analogue for 96 hours. p53 siRNA significantly inhibited the dose dependent induction of p53 (Fig. 4B).

p53 siRNA reduces sensitivity of MCF-7 cells to polyamine analogue-induced growth inhibition and apoptosis. MCF-7 cells transfected with empty vector or p53 siRNA were treated with increasing concentrations of each analogue (1–10 μ M) for 96 h followed by MTT growth inhibition assays to determine if the expression of the p53 siRNA could alter the sensitivity of tumor cells to polyamine analogue cytotoxicity. MCF-7 cells transfected with p53 siRNA exhibited more resistance to growth inhibition by each analogue at concentrations above 1 μ M (Fig. 5A). This was associated with the inhibition of apoptotic internucleosomal DNA fragmentation by p53 siRNA in CGC-11144 treated MCF-7 cells (Fig. 5B). These data suggests that activation of p53 is a common mechanism that may contribute to growth inhibition and apoptotic cell death induced by these structurally distinct analogues.

Effects of p53 siRNA on cell cycle regulatory proteins. p53 siRNA significantly inhibited the dose dependent induction of p21 (Fig. 6A) by each analogue, demonstrating that activation of p21 by polyamine analogues occurs through p53 dependent mechanisms in MCF-7 cells. Downregulation of cyclin D1 and cyclin B1 by oligoamines was not blocked by p53 siRNA transfection (Fig. 6A). However, p53 siRNA prevented the decrease in cyclin B1 levels induced by BENSpM and CHENSpM. CDK activity was assessed by kinase assays following immunoprecipitation from cell extracts

harvested from vector or p53 siRNA-transfected MCF-7 cells treated with specific polyamine analogues at 10 μ M for 96 h. The oligoamines, CGC-11144 and CGC-11172, significantly inhibit CDK4, CDK6 and CDK2 kinase activities in vector-transfected control cells (Fig. 6B), whereas BENSpM decreased only CDK2 activity and CHENSpM did not modulate the activities of any of the examined kinases. Expression of p53 siRNA completely blocked the downregulation of CDK4 activity and partially blocked the decreased CDK6 activity by oligoamines. However, the inhibition of CDK2 activity by CGC-11144, CGC-11172 and BENSpM was not affected by the knockdown of p53 expression. In addition, the CDC2 (CDK1) activity was not changed by any of the polyamine analogues in either vector or p53 siRNA transfectants (data not shown).

Effects of p53 siRNA on polyamine analogue induced cell cycle arrest. To assess the effects of siRNA-mediated downregulation of the p53/p21 pathway on polyamine analogue-induced cell cycle arrest, vector and p53 siRNA transfectants were treated with 10 μ M of each analogue for 96 h followed by flow cytometric analysis. Figure 7 shows that suppression of analogue-mediated p53/p21 induction by siRNA has little effect on oligoamine-induced G_1 arrest. However, the BENSpM-induced G_1 arrest was almost completely reversed by p53 siRNA transfection. CHENSpM treatment of vector transfectants resulted in significant G_2/M arrest and induction of a large fraction of cells detected as a sub- G_1 peak, which is believed to represent an apoptotic cell population. Expression of p53 siRNA considerably reduced the fraction of apoptotic cells induced by CHENSpM without altering the proportion of cells at G_1 . However, an increase of cells in G_2/M was observed in p53 siRNA transfectants treated by CHENSpM, suggesting that p53 siRNA suppresses CHENSpM-induced apoptosis without affecting CHENSpM's effect on cell cycle arrest at G_2/M phase. Together, these data indicate that the p53/p21 signaling pathway may mediate polyamine analogue-induced cell cycle arrest through agent-specific mechanisms.

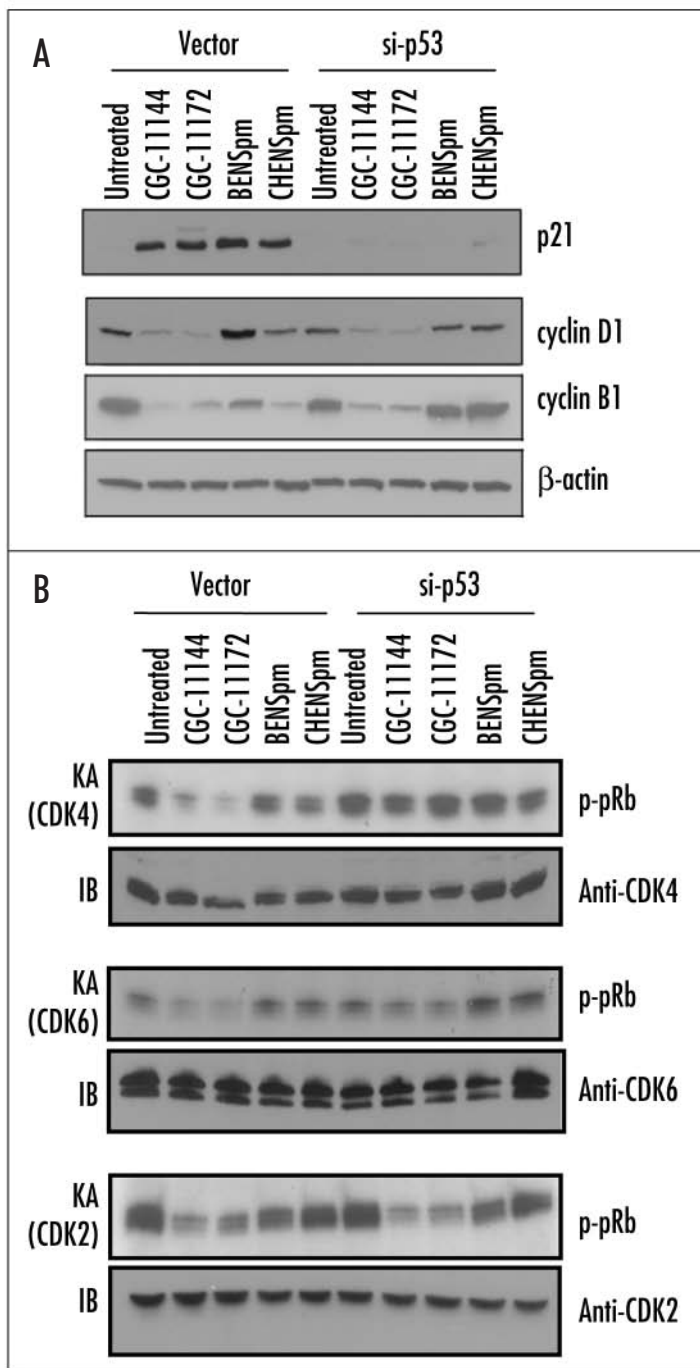


Figure 6. Effects of p53 siRNA on cell cycle regulatory proteins. (A) Vector and p53 siRNA transfected cells were treated with 10 μ M of polyamine analogue for 96 h and subjected to immunoblotting with an antibody to p21, cyclin D1 or cyclin B1. Actin protein was blotted as a control. Each experiment was performed twice with similar results. (B) CDKs complexes were immunoprecipitated with anti-CDKs antibody and then subjected to an in vitro kinase assay (KA) using pRb as the substrate. CDK protein expression was analyzed by Western blotting as a control (IB).

DISCUSSION

Polyamine analogues chosen for this study represent three major classes of analogues. All four analogues inhibited the in vitro growth of MCF-7 cells at micromolar concentrations. Previous studies have demonstrated that natural polyamines are required for cell cycle

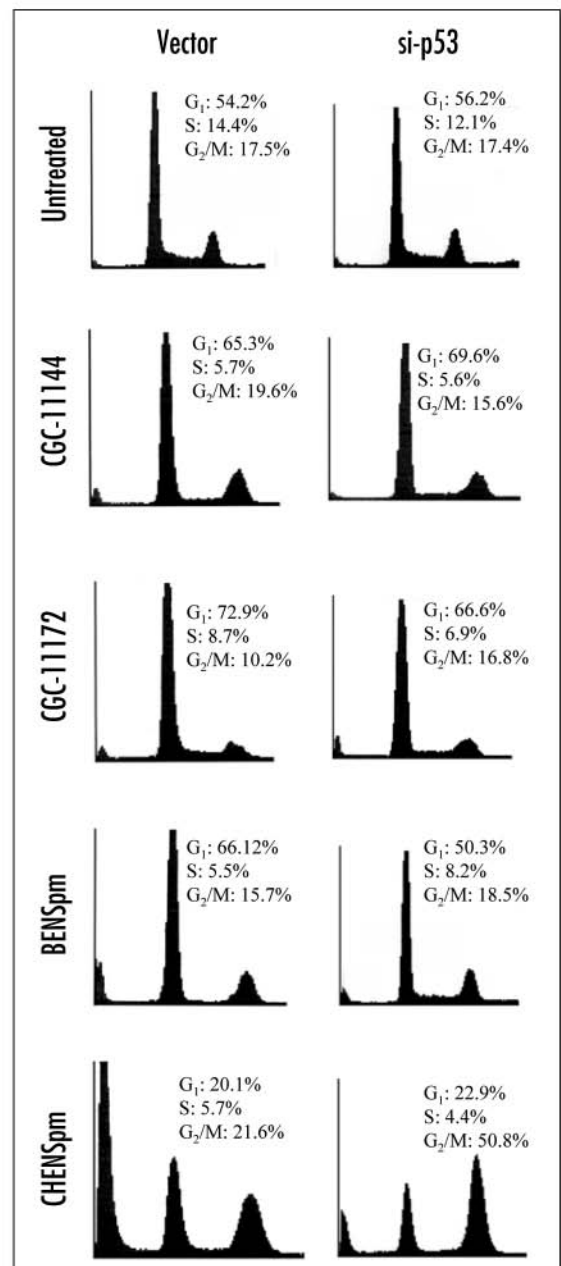


Figure 7. Effects of p53 siRNA on polyamine analogue induced cell cycle arrest. Vector and p53 siRNA transfected MCF-7 cells were treated with 10 μ M of polyamine analogue for 96 h. After treatment, cells were harvested and stained for DNA with Hoechst 33258 for flow cytometric analysis. The fractions corresponding to G₁, S and G₂/M phases of the cell cycle are indicated. Each experiment was performed twice with similar results.

checkpoint regulation.²⁵ Polyamines are essential for cells to enter S-phase and depletion of intracellular polyamines may lead to the inhibition of G₁-S phase transition.²⁶ In this study, oligoamines and BENSpm blocked the cell cycle at G₁ phase, while CHENSpm produced a significant G₂/M arrest in MCF-7 cells (Fig. 2B). Interestingly, all three polyamine analogues capable of G₁ arrest are symmetrical, which is consistent with previous reports in lung cancer and melanoma cells.^{27,28} The mechanism underlying this observation is not clear. During the cell cycle, ODC activity increases during G₁ phase and reaches a second peak at G₂ phase prior to mitosis.²⁹ Studies by several other groups have demonstrated that inhibition of

ODC by DFMO led to G₁ arrest in a variety of cell lines including tumor cells.³⁰⁻³² Both oligoamines and BENSp_m profoundly decrease ODC activity within 24 h in MCF-7 cells, potentially through the activation of the ODC inhibitor, antizyme (AZ),^{18,33} implying that downregulation of ODC could be one mechanism leading to G₁ arrest. In contrast, CHENSp_m differs from BENSp_m only at the terminally modified cycloheptylmethyl group, but leads to a G₂/M arrest. A previous study found that CHENSp_m altered microtubule density and polymerization in lung cancer cells.²⁷ The mechanism underlying this effect is still under investigation. However, these findings suggest that small changes in analogue structure may lead to profound difference in cellular effects and the ability of analogues to alter cell cycle transition and tubulin polymerization.

That all four polyamine analogues tested induce p53/p21 in MCF-7 cells indicates that activation of p53/p21 is a common response of MCF-7 cells to polyamine analogues with different structures. The p53 tumor suppressor gene is a central mediator of apoptotic and cell cycle response to a variety of stimuli such as DNA damage, hypoxia, genotoxic stress, and ribonucleoside triphosphate deficiency.³⁴ BENSp_m-induced p53/p21 activation and G₁ arrest has been reported in human melanoma cells,¹⁵ indicating that p53/p21 might play an active role in the mediation of polyamine analogue-induced cell cycle arrest and growth inhibition in multiple types of cancer. Specific siRNA targeting p53 mRNA significantly reduced the sensitivity of MCF-7 cells to polyamine analogue-induced growth inhibition and apoptosis. These results support our preliminary hypothesis that activation of the p53/p21 signaling pathway may contribute to polyamine analogue cytotoxicity in human breast cancer cells.

Our data also demonstrated that the effect of p53/p21 on polyamine analogue-induced cell cycle arrest exhibits a complicated pattern that is agent-dependent. Suppression of p53/p21 was not sufficient to reverse the G₁ cell cycle arrest induced by oligoamines. This finding implies that p53 induced growth inhibition and apoptosis may occur through cell cycle arrest-independent pathway(s). Activation of the cyclin-dependent kinase inhibitor, p21, plays a critical role in the induction of cell cycle arrest by p53, but p21 does not appear to be a p53-targeted apoptotic gene, like Bax, Fas, Noxa, DR5, etc.³⁵ Also, although oligoamine-inhibited CDK4 and CDK6 activities were restored by p53 knockdown, expression of p53 specific siRNA did not alter the inhibition of CDK2 activity or the down-regulation of cyclin D1 and cyclin B1 protein expression by oligoamines (Fig. 6), suggesting that p53-independent mechanisms exist in oligoamine-induced cell cycle arrest. That oligoamines can also cause the G₁ arrest in p53 mutant breast cancer cells like MDA-MB231 (data not shown) further suggests that oligoamines may induce cell cycle arrest via some p53-independent mechanisms. Perhaps, the strong interaction between oligoamine and DNA molecules may cause more severe damage than that seen with other analogues and G₁ arrest due to such profound DNA damage may not be overcome by inhibition of the CDK inhibitor activities only. Conversely, BENSp_m-induced G₁ arrest was reversed by p53 siRNA, indicating that this effect is more dependent on the p53/p21 pathway than other analogues. It is possible that BENSp_m may exert cytotoxicity largely by superinduction of SSAT activity, rather than direct interaction with DNA. Suppression of p53/p21 activation significantly inhibited CHENSp_m-induced apoptosis but did not affect the accompanying G₂/M arrest (Fig. 7). A previous study has demonstrated that CHENSp_m induces G₂/M arrest by altering tubulin dynamics.²⁷ The effect of CHENSp_m on microtubules could trigger

the activation of downstream apoptotic pathways that are regulated by p53/p21 signaling pathway. Suppression of p53 activation by siRNA could selectively inhibit the apoptotic pathway without affecting the ability of CHENSp_m to change tubulin dynamics and induce G₂/M arrest. In aggregate, these results suggest that the role of the p53/p21 pathway in polyamine analogue-induced cell cycle arrest varies by agent and greatly depends on the balance between the extent of DNA damage and the response of repair/apoptotic mechanisms.

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Spermine Oxidase SMO(PAOh1), Not N^1 -Acetylpolyamine Oxidase PAO, Is the Primary Source of Cytotoxic H_2O_2 in Polyamine Analogue-treated Human Breast Cancer Cell Lines*

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Allison Pledgie[‡], Yi Huang[‡], Amy Hacker[‡], Zhe Zhang[‡], Patrick M. Woster[§], Nancy E. Davidson^{‡1}, and Robert A. Casero, Jr.^{‡2}

From the [‡]Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins University, Baltimore, Maryland 21231 and the

[§]Department of Pharmaceutical Sciences, College of Pharmacy and Allied Health Professions, Wayne State University, Detroit, Michigan 48202

The induction of polyamine catabolism and its production of H_2O_2 have been implicated in the response to specific antitumor polyamine analogues. The original hypothesis was that analogue induction of the rate-limiting spermidine/spermine N^1 -acetyltransferase (SSAT) provided substrate for the peroxisomal acetylpolyamine oxidase (PAO), resulting in a decrease in polyamine pools through catabolism, oxidation, and excretion of acetylated polyamines and the production of toxic aldehydes and H_2O_2 . However, the recent discovery of the inducible spermine oxidase SMO(PAOh1) suggested the possibility that the original hypothesis may be incomplete. To examine the role of the catabolic enzymes in the response of breast cancer cells to the polyamine analogue N^1,N^1 -bis(ethyl)norspermine (BENSpm), a stable knockdown small interfering RNA strategy was used. BENSpm differentially induced SSAT and SMO(PAOh1) mRNA and activity in several breast cancer cell lines, whereas no N^1 -acetylpolyamine oxidase PAO mRNA or activity was detected. BENSpm treatment inhibited cell growth, decreased intracellular polyamine levels, and decreased ornithine decarboxylase activity in all cell lines examined. The stable knockdown of either SSAT or SMO(PAOh1) reduced the sensitivity of MDA-MB-231 cells to BENSpm, whereas double knockdown MDA-MB-231 cells were almost entirely resistant to the growth inhibitory effects of the analogue. Furthermore, the H_2O_2 produced through BENSpm-induced polyamine catabolism was found to be derived exclusively from SMO(PAOh1) activity and not through PAO activity on acetylated polyamines. These data suggested that SSAT and SMO(PAOh1) activities are the major mediators of the cellular response of breast tumor cells to BENSpm and that PAO plays little or no role in this response.

The natural polyamines, spermine, spermidine, and putrescine, are ubiquitous polycationic alkylamines that are required for normal eukaryotic cell growth and differentiation (1, 2). Neither mammalian cells lacking polyamine biosynthetic enzymes nor cells depleted of poly-

amines are able to replicate (3). Polyamine metabolism is frequently dysregulated in many types of cancer, including breast, prostate, and lung cancer (1, 4–6). Consequently, the polyamine metabolic pathway has become an attractive target for the development of antineoplastic agents (5, 7, 8).

Although early work focused on developing drugs that inhibited polyamine biosynthesis, more recent attention has been given to polyamine analogues that, in addition to down-regulating biosynthesis, also up-regulate polyamine catabolism (9–14). Until recently, mammalian intracellular polyamine catabolism was considered to be a consequence of two enzymes, the rate-limiting and inducible cytosolic spermidine/spermine N^1 -acetyltransferase (SSAT)³ and a relatively constitutively expressed, peroxisomal N^1 -acetylpolyamine oxidase (PAO) (1, 2). The products of SSAT/PAO activities on spermine and spermidine are the reactive oxygen species, H_2O_2 , spermidine, and putrescine, respectively (depending on the starting substrate), and 3-acetoaminopropanol. The activity of the SSAT/PAO pathway has been linked previously with the cytotoxic response of several tumor types to specific polyamine analogues (10, 15–17). However, recent studies have clearly demonstrated that an additional enzyme exists in the mammalian polyamine catabolic pathway, an inducible spermine oxidase (SMO/PAOh1) (18, 19). SMO(PAOh1) is a cytosolic protein that is selectively active on spermine producing H_2O_2 , spermidine, and the aldehyde 3-aminopropanol (20, 21). More importantly, the expression of this enzyme is induced by some of the same agents that induce SSAT, suggesting that induction of both of the polyamine catabolic pathways can lead to the production of H_2O_2 (22).

Because the production of H_2O_2 through polyamine catabolism has been implicated in the cytotoxic response of several tumor types to multiple polyamine analogues, the purpose of this study was to determine the origin of H_2O_2 in response to cellular exposure to the antitumor polyamine analogue, N^1,N^1 -bis(ethyl)norspermine (BENSpm) (an agent that has been evaluated in phase I and II clinical trials), and thereby to determine the role of each of the polyamine catabolic enzymes in the BENSpm response (23, 24). Previous studies implicating PAO in H_2O_2 production in response to analogue exposure were performed with the polyamine oxidase inhibitor, MDL72527, thought to be

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¹ To whom correspondence may be addressed: The Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins University, 1650 Orleans St., CRB 409, Baltimore, MD 21231. Tel.: 410-955-8489; Fax: 410-614-4073; E-mail: davidna@jhmi.edu.

² To whom correspondence may be addressed: The Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins University, 1650 Orleans St., CRB 551, Baltimore, MD 21231. Tel.: 410-955-8580; Fax: 410-614-9884; E-mail: rcasero@jhmi.edu.

³ The abbreviations used are: SSAT, spermidine/spermine N^1 -acetyltransferase; SMO(PAOh1), spermine oxidase; BENSpm, N^1,N^1 -bis(ethyl)norspermine; ODC, ornithine decarboxylase; AdoMetDC, S-adenosylmethionine decarboxylase; PAO, N^1 -acetylpolyamine oxidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA; FACS, fluorescence-activated cell sorter; CM- H_2 DCFDA, 5-(and -6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate; AT, 3-amino-1,2,4-triazole; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

specific for PAO. With the recognition that MDL72527 is also a potent inhibitor of SMO(PAO1), the results of earlier studies may require re-examination (25–27). Also, previous attempts to examine directly the role of SSAT in cellular response through the use of siRNA strategies were limited by a transient transfection approach, thus making it difficult to assess long term effects of extended SSAT knockdown in response to analogue treatment (28). To overcome the limitations of transient knockdown, a stable transfection strategy was used to constitutively express siRNAs targeting the rate-limiting steps of polyamine catabolism, SSAT and SMO(PAOh1), either alone or in combination.

This study represents the first use of stably expressed siRNAs directed against multiple key polyamine metabolic enzymes and demonstrates that SSAT and SMO(PAOh1) induction contribute significantly to the antiproliferative effects of BENSpm in a cell type-specific manner. Furthermore, the experimental results confirm that SMO(PAOh1) enzyme activity, not PAO enzyme activity, is the source of cytotoxic H₂O₂ produced followed exposure to BENSpm in specific breast cancer lines, whereas SSAT induction results in a decrease in intracellular polyamine levels through the acetylation of polyamines that are then exported from the cell. With a better understanding of the relative contribution made by each of the independent polyamine catabolic pathways to the cytotoxic activity of polyamine analogues, it is hoped that more selective and effective agents can be designed for use against breast cancer.

EXPERIMENTAL PROCEDURES

Cell Lines, Culture Conditions, and Reagents—The acquisition and maintenance of the breast cancer cell lines, MDA-MB-231, Hs578t, MCF-7, and T47D, have been described previously (29). BENSpm and MDL72527 were synthesized as described previously (14, 30). 5-(and -6)-Chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA), mixed isoforms, was purchased from Molecular Probes (Eugene, OR). Catalase and 3-amino-1,2,4-triazole (AT) were purchased from Sigma.

RNA Isolation, Reverse Transcription-PCR, and Real Time PCR—Total cellular RNA was isolated from cultured cell lines using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 3 µg of total RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen) and oligo(dT) primers (Invitrogen). Conventional PCR was performed using the cDNA as a template with the following primers: SSAT forward, 5'-ATCTAAGC-CAGGTTGCAATGA, and SSAT reverse, 5'-GCACTCCTCACTCC-TCTGTTG; SMO(PAOh1) forward, 5'-CGCAGACTTACTTCCCC-GGC, and SMO(PAOh1) reverse, 5'-CGCTCAATTCCTCAACC-ACG; SMO(PAOh1) isoform 1 forward, 5'-CGACCACAATCACGA-CACTG, and SMO(PAOh1) and isoform 1 reverse, 5'-GCCGAGGG-CAAGATTCGCCG; SMO(PAOh1) isoform 2 forward, 5'-GCCCCG-GGGTGTGCTAAAGAG, and SMO(PAOh1) and isoform 2, reverse 5'-CGGAAAACAGCACCTGCATGG; SMO(PAOh1) isoform 3 forward, 5'-CGCAGACTTACTTCCCCGGCTCAG, and SMO(PAOh1) isoform 3 reverse, 5'-CTGCATGGGCTCGTTGTATAAATC; SMO-(PAOh1) isoform 4 forward, 5'-CCAGGCCTCAGCCCGCCCCAG, and SMO(PAOh1) isoform 4 reverse, 5'-GCTGTTCTGGGAAGTTG-GAAGAG; PAO forward, 5'-CCTACAGTTTGTGTGGGAGGA, and PAO reverse, 5'-ATGAATAGGAGCCACGGAAGT; actin forward, 5'-ACCATGGATGATGATGATATCGC and actin reverse, 5'-ACA-TGGCTGGGGTCTGAAG. PCR products were resolved by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. For real time PCR, cDNA was amplified by using SYBR green (Sigma) according to the manufacturer's instructions. Real time PCR data were

acquired and analyzed using Sequence Detector version 1.7 software (PerkinElmer Life Sciences) and were normalized to the GAPDH house-keeping gene.

Analysis of Intracellular Polyamine Levels and Enzyme Activities—The polyamine content of treated and untreated cells was determined by using the precolumn dansylation, reversed phase high pressure liquid chromatography method as described previously using 1, 7-diaminoheptane as the internal standard (31). SSAT and ODC activities were determined by using ¹⁴C-labeled substrates and scintillation counting of end products produced as described previously (12). The enzyme activities of SMO(PAOh1) and PAO in cell lysates were assayed as described previously by using 250 µM spermine (Sigma) or N¹-acetylspermine (Sigma), respectively, as the substrate (18). Protein concentrations were determined by using the Bradford method (32).

Cell Growth and MTT Assays—Cells were plated at a cell density of 5,000 cells/well in 6-well tissue culture plates. After attachment overnight, the medium was replaced, and cells were incubated with or without 10 µM BENSpm for up to 96 h. Every 24 h, cells were detached by trypsinization and counted using a Coulter particle counter. MTT assays were performed as described previously (29). Briefly, following attachment overnight, cells were incubated with increasing concentrations of BENSpm in the presence or absence of 25 µM MDL72527 for 96 h. All of the experiments were plated in quadruplicate and were performed three times. The results from the MTT assays were validated by direct comparison to a conventional cell growth assay.

Flow Cytometry—MDA-MB-231 and MCF-7 cells were plated at a cell density of 100,000 cells in 10-cm culture dishes and were treated with 10 µM BENSpm for up to 96 h. Adherent and nonadherent cells were collected, sedimented at 200 × g for 10 min, washed with ice-cold phosphate-buffered saline, fixed with 4.44% formaldehyde (Sigma), and stained with Hoechst 33258 (Sigma). BD-LSR (BD Biosciences) was used to perform FACS, and the cell cycle was analyzed using Cell-Quest software (BD Biosciences).

RNA Interference and Transfections—The SMO(PAOh1) stable siRNA clones were generated by annealing and inserting the following oligonucleotides (Invitrogen) into the pSilencer 2.1-U6 neo expression vector (Ambion, Austin, TX) according to the manufacturer's instructions: SMO(PAOh1) forward, 5'-GAT CCG CAC TTC TTG AGC AGG GTT TTC AAG AGA AAC CCT GCT CAA GAA GTG CTT TTT TGG AAA, and SMO(PAOh1) reverse, 5'-AGC TTT TCC AAA AAA GCA CTT CTT GAG CAG GGT TTC TCT TGA AAA CCC TGC TCA AGA AGT GCG. The following oligonucleotides (Invitrogen) targeting the SSAT gene were annealed to form the hairpin siRNA template insert that was then ligated into the pSilencer 2.1-U6 hygro expression vector (Ambion) according to the manufacturer's instructions: SSAT forward, 5'-GAT CCG TGA TCC TCC CAC CTC AGC TTC AAG AGA GCT GAG GTG GGA GGA TCA CTT TTT TGG AAA, and SSAT reverse, 5'-AGC TTT TCC AAA AAA GTG ATC CTC CCA CCT CAG CTC TCT TGA AGC TGA GGT GGG AGG ATC ACG.

Lipofectamine was used to transfect 4 µg of the targeting plasmid or provided nonsense control plasmid (Ambion) into MDA-MB-231 and MCF-7 cells. Single clones representing MDA-MB-231 nonsense vector control, MDA-MB-231 ΔSMO(PAOh1) (SMO(PAOh1) stably knocked down alone), MDA-MB-231 ΔSSAT (SSAT stably knocked down), MDA-MB-231 ΔSSAT/ΔSMO(PAOh1) (both SMO(PAOh1) and SSAT stably knocked down), MCF-7 nonsense vector control, MCF-7 ΔSMO(PAOh1) (SMO(PAOh1) stably knocked down), MCF-7 ΔSSAT (SSAT stably knocked down), and MCF-7 ΔSSAT/ΔSMO(PAOh1) (both SMO(PAOh1) and SSAT stably knocked down) were chosen.

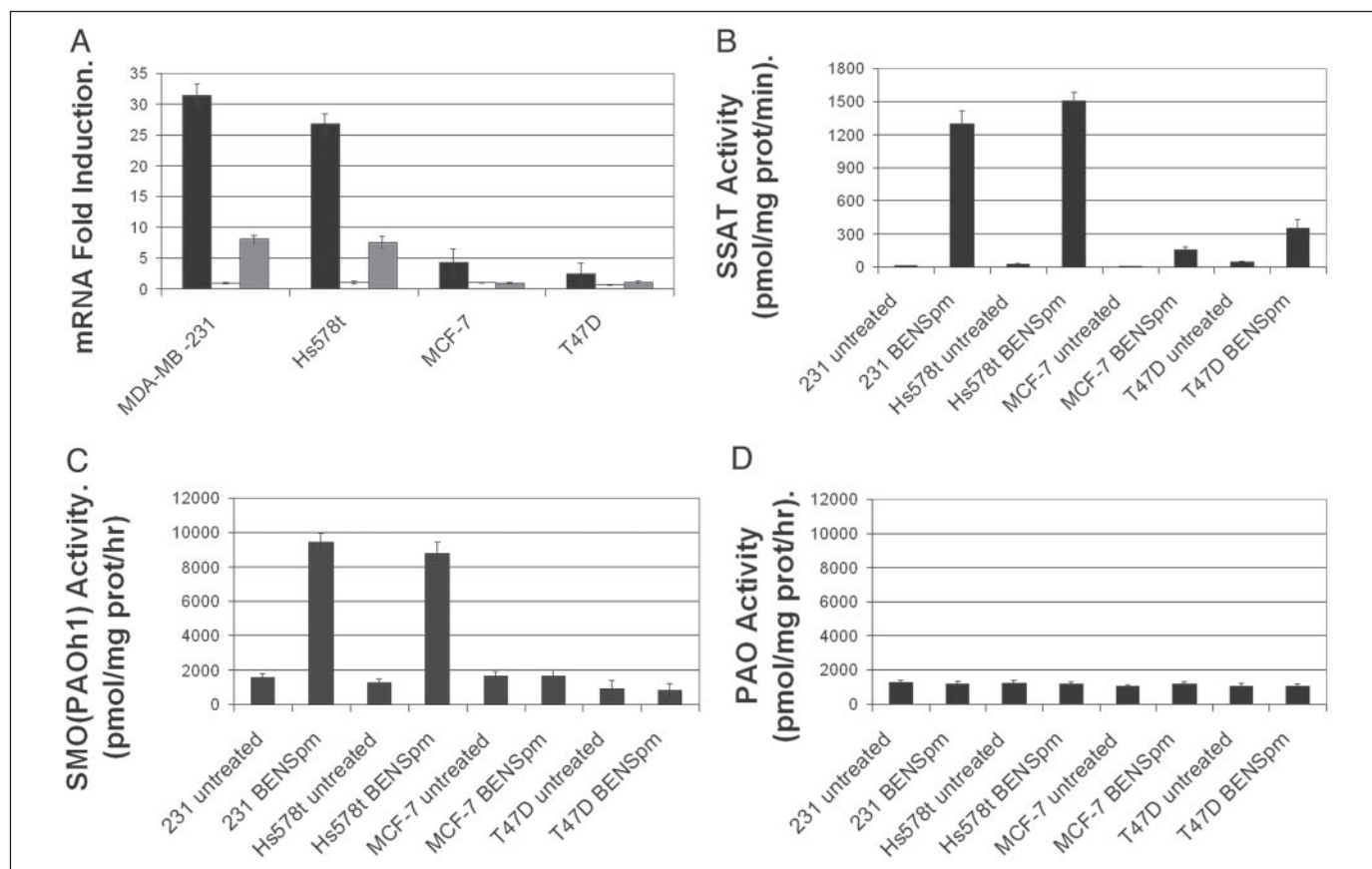


FIGURE 1. BENSpm induces SSAT and SMO(PAOh1) mRNA and activity in multiple human breast cancer cell lines. MDA-MB-231, Hs578t, MCF-7, and T47D breast cancer cell lines were treated with $10 \mu\text{M}$ BENSpm for 24 h. *A*, real time PCR for SSAT mRNA (black bars), PAO mRNA (white bars), and SMO(PAOh1) mRNA (gray bars) was performed as described under "Experimental Procedures"; all values were normalized to the GAPDH housekeeping gene. Values are the means \pm S.D. of four independent experiments performed in duplicate. *B*, SSAT activity was determined as described under "Experimental Procedures." *C*, SMO(PAOh1) activity was assayed as described under "Experimental Procedures" using $250 \mu\text{M}$ spermine as the substrate. *D*, PAO activity was determined as described under "Experimental Procedures" using $250 \mu\text{M}$ N^1 -acetylspermine as the substrate. Enzyme activity values are the means \pm S.D. of three independent experiments performed in triplicate.

Clones were selected and maintained in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) supplemented with 5% fetal bovine serum (Mediatech), 1% glutamine (Mediatech), and $500 \mu\text{g/ml}$ G418 (Sigma) or $500 \mu\text{g/ml}$ hygromycin (Roche Applied Science) as required. All data presented here are the average of multiple, independent experiments performed using at least three clones for each cell type.

Measurement of H_2O_2 .—Cells were treated for 24 h with $10 \mu\text{M}$ BENSpm with or without co-treatment of $25 \mu\text{M}$ MDL72527 or 500 units/ml catalase, and intracellular H_2O_2 was detected by FACS. Following treatment, adherent cells were harvested with trypsin and were combined with nonadherent cells. Cells were washed with $1\times$ phosphate-buffered saline (Mediatech), and 1×10^6 cells were treated with $10 \mu\text{M}$ CM- H_2 DCFDA for 30 min at 37°C . Ten thousand cells were then analyzed by FACS on a BD-LSR (BD Biosciences) as reported previously (33).

Statistical Methods.—For cell growth assays, the longitudinal data were analyzed using a mixed effects model that accounts for the correlation among repeated measurements (Fig. 2). An exchangeable covariance structure was assumed in the mixed effects model, and cell growth data were fit with a quadratic growth curve model (Fig. 2). Analysis of variance was used to examine the changes in SSAT and SMO(PAOh1) mRNA and activity in BENSpm-treated cell lines (Figs. 3 and 4). Bonferroni adjustment was applied for multiple comparisons. Analysis of covariance was used to examine the difference in the sensitivity to BENSpm among the treated cell lines while controlling for treatment concentration effect (Fig. 5). Pairwise least square means were com-

pared when the overall difference among cell lines was observed. *p* values were not adjusted for multiple comparisons in the analysis of this experiment (Fig. 5). A *p* value of ≤ 0.05 was considered a statistically significant difference between compared groups. All analyses were conducted with SAS System software (version 9.1).

RESULTS

BENSpm Induces SSAT and SMO(PAOh1) mRNA and Activity in Multiple Human Breast Cancer Cell Lines.—The induction of SSAT, PAO, and SMO(PAOh1) mRNA and activity by BENSpm was examined in four breast cancer cell lines that represent a wide range of breast cancer phenotypes, MDA-MB-231, Hs578t, MCF-7, and T47D cells. Following treatment with $10 \mu\text{M}$ BENSpm for 24 h, real time PCR was used to examine changes in SSAT, PAO, and SMO(PAOh1) mRNA (Fig. 1A). SSAT mRNA was induced in all four cell lines following BENSpm exposure, although induction of SMO(PAOh1) mRNA was only seen in MDA-MB-231 and Hs578t cells; no PAO mRNA induction was detected in any cell line. Changes in enzyme activity for each of the catabolic enzymes were then examined in each cell line following the same treatment. SSAT enzyme activity was induced in each cell line with the greatest induction observed in BENSpm-treated MDA-MB-231 and Hs578t cells (Fig. 1B). The induction of SMO(PAOh1) enzyme activity closely correlated with the induction of mRNA with induction only seen in MDA-MB-231 and Hs578t cells (Fig. 1C), although no induction of PAO enzyme activity was detected in any of the breast cancer cell lines examined (Fig. 1D). No significant expression or induc-

FIGURE 2. BENSpm inhibits the growth of multiple breast cancer cell lines. MDA-MB-231, Hs578t, MCF-7, and T47D breast cancer cell lines were treated with 10 μ M BENSpm for 96 h. Cells were detached with trypsinization and were counted every 24 h as described under "Experimental Procedures." The results are the means \pm S.D. of three independent experiments performed in triplicate with $p < 0.001$ after 48 h of BENSpm treatment in each cell line as determined using a mixed effects model.

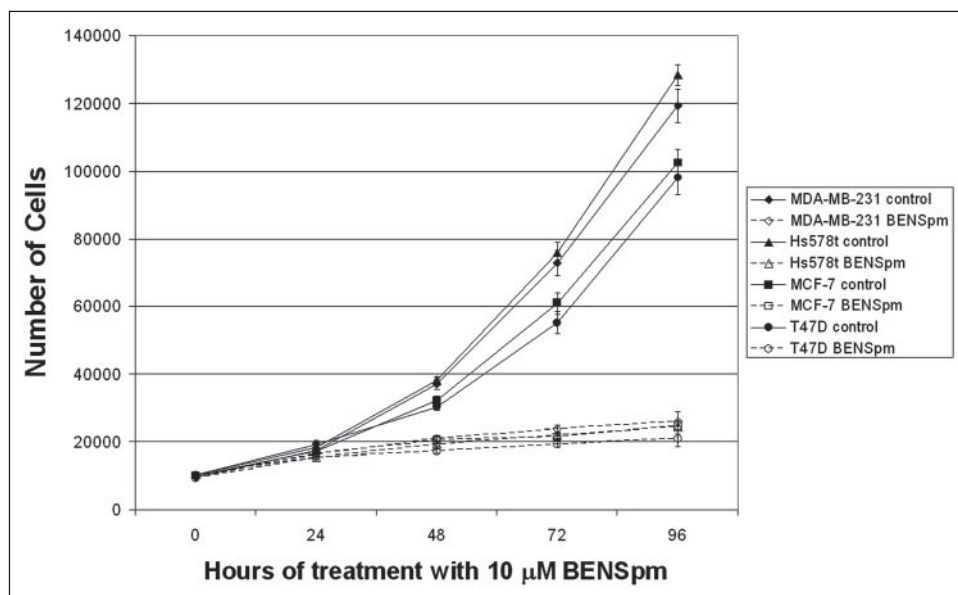


TABLE ONE

Effects of BENSpm treatment on intracellular polyamine levels and ODC activity

MDA-MB-231, Hs578t, MCF-7, and T47D cells were treated with 10 μ M BENSpm for 24 h. Intracellular polyamine levels and ODC enzyme activity were assayed as described under "Experimental Procedures." Values are the means \pm S.D. of three independent experiments in duplicate for the polyamines and three independent experiments in triplicate for ODC enzyme activity.

Cell line	Treatment	Polyamines			BENSpm	ODC activity
		Putrescine	Spermidine	Spermine		
		nmol/mg protein			nmol/mg protein	pmol CO ₂ /mg protein/h
MDA-MB-231	Control	3.9 \pm 0.2	36.9 \pm 4.6	47.0 \pm 8.2	ND	577.9 \pm 26.1
	BENSpm	2.3 \pm 0.3	10.9 \pm 1.2	20.6 \pm 3.4	38.5 \pm 4.4	76.7 \pm 4.8
Hs578t	Control	2.6 \pm 0.7	46.6 \pm 3.2	45.3 \pm 2.9	ND	480.6 \pm 19.5
	BENSpm	1.2 \pm 0.2	15.7 \pm 1.6	22.2 \pm 1.1	45.2 \pm 4.7	93.4 \pm 9.2
MCF-7	Control	6.2 \pm 0.3	65.8 \pm 6.9	52.2 \pm 7.8	ND	1525.7 \pm 116.6
	BENSpm	2.7 \pm 0.2	26.8 \pm 5.4	26.4 \pm 4.7	52.3 \pm 5.9	93.4 \pm 12.4
T47D	Control	4.3 \pm 0.5	60.7 \pm 4.4	47.0 \pm 2.8	ND	1264.6 \pm 65.3
	BENSpm	2.2 \pm 0.4	26.2 \pm 1.7	14.7 \pm 0.9	42.6 \pm 2.4	103.1 \pm 6.5

tion of SMO(PAOh1) or SSAT was seen in an immortalized nontumorigenic mammary epithelial cell line, MCF-10A, suggestive of a behavior similar to MCF-7 cells (data not shown).

BENSpm Inhibits Cell Growth, Reduces Intracellular Polyamine Levels, and Reduces ODC Enzyme Activity in Several Human Breast Cancer Cell Lines—The effect of BENSpm on breast cancer cell growth was examined by treating MDA-MB-231, Hs578t, MCF-7, and T47D cells with 10 μ M BENSpm for 96 h. Treatment of each cell line with 10 μ M BENSpm for \geq 48 h significantly inhibited cell growth (Fig. 2). Cell growth in each cell line was inhibited similarly by BENSpm through 96 h of exposure. FACS analysis showed no difference in cell cycle staging in BENSpm treated MDA-MB-231 and MCF-7 cells; both cell lines arrested in G₁ phase after 48 h of BENSpm treatment and remained in a G₁ block through 96 h (data not shown). All four breast cancer cell lines treated with 10 μ M BENSpm for 24 h exhibited a similar decrease of \sim 50% in the levels of spermine, spermidine, and putrescine upon BENSpm treatment with a similar level of BENSpm accumulation in each cell line (TABLE ONE). BENSpm treatment also reduced ODC enzyme activity between 5- and 16-fold in each cell line (TABLE ONE).

Selection and Validation of Δ SSAT, Δ SMO(PAOh1), and Δ SSAT/ Δ SMO(PAOh1) Clones—To assess the relative role played by SSAT and SMO(PAOh1) in determining BENSpm response, RNA interference

was used to knock down the expression of each enzyme, alone and in combination, in MDA-MB-231 and MCF-7 cells. These cell lines were chosen as they are representative of both hormone-insensitive and hormone-sensitive breast cancers, respectively, and display different enzyme responses to BENSpm. All data presented here are averages of multiple, independent experiments performed using three clones for each cell type. The knockdown of SSAT and SMO(PAOh1) mRNA and enzyme activity in MDA-MB-231 and MCF-7 cells was confirmed by real time PCR and activity assays (Fig. 3). The SSAT and SMO(PAOh1) siRNAs used were both specific and efficient in reducing the related mRNA and activity levels. Furthermore, the knockdown of SMO(PAOh1) in MDA-MB-231 and MCF-7 cells did not significantly alter the induction of SSAT mRNA or activity in either cell line. Similarly, knocking down SSAT in MDA-MB-231 cells did not significantly affect the induction of SMO(PAOh1) mRNA or activity. In the absence of drug treatment, the growth of MDA-MB-231 and MCF-7 cells was not altered by the knockdown of either enzyme alone or in combination (data not shown).

The Knockdown of SSAT Reduces BENSpm-induced Intracellular Polyamine Depletion but Has No Effect on BENSpm-induced Down-regulation of ODC Enzyme Activity—Intracellular and extracellular polyamines were then measured to examine the effects of the knock-

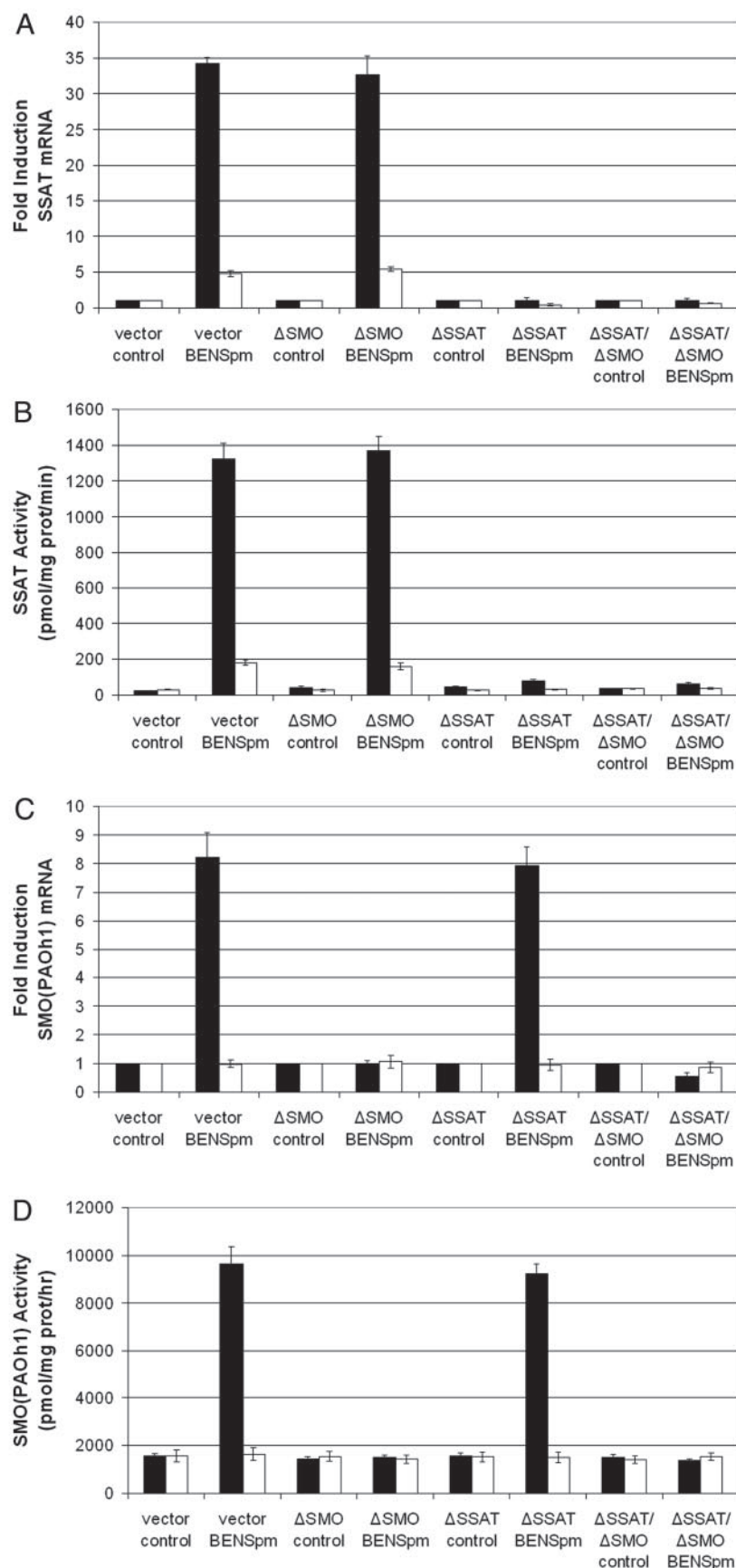


FIGURE 3. siRNA directed against SMO(PAOh1) and SSAT specifically and efficiently reduces respective SSAT and SMO(PAOh1) mRNA and activity induction by BENSpm. Transfected MDA-MB-231 cells (black bars) and MCF-7 cells (white bars) were treated with 10 μ M BENSpm for 24 h. Real time PCR for SSAT mRNA (A) and real time PCR for SMO(PAOh1) mRNA (C) was performed as described under "Experimental Procedures"; all values were normalized to the GAPDH housekeeping gene. Values are the means \pm S.D. of four independent experiments performed in duplicate. B, SSAT enzyme activity was assayed as described under "Experimental Procedures." D, SMO(PAOh1) enzyme activity was assayed as described under "Experimental Procedures" using 250 μ M spermine as the substrate. Enzyme activity values are the means \pm S.D. of three independent experiments performed in triplicate. Analysis of variance demonstrated that the induction of SSAT mRNA and activity with BENSpm in MDA-MB-231 vector and MDA-MB-231 Δ SMO(PAOh1) cells was significantly different from BENSpm-treated MDA-MB-231 Δ SSAT cells and MDA-MB-231 Δ SSAT/ Δ SMO(PAOh1) cells ($p < 0.0001$ and $p < 0.0001$, respectively). BENSpm-induced SSAT mRNA and activity in MCF-7 vector and MCF-7 Δ SMO(PAOh1) cells were significantly different from BENSpm-treated MCF-7 Δ SSAT and MCF-7 Δ SSAT/ Δ SMO(PAOh1) cells ($p < 0.0001$ and $p < 0.0001$, respectively). BENSpm-treated MDA-MB-231 vector and MDA-MB-231 Δ SSAT cells had a similar induction of SMO(PAOh1) mRNA and activity ($p = 0.069$ and $p = 0.114$, respectively) that was significantly different from BENSpm-treated MDA-MB-231 Δ SMO(PAOh1) and MDA-MB-231 Δ SSAT/ Δ SMO(PAOh1) cells ($p < 0.0001$ and $p < 0.0001$, respectively).

TABLE TWO

Effects of BENSp^m treatment on intracellular and extracellular polyamine levels

Cells were treated with 10 μ M BENSp^m for 24 h. Intracellular polyamine levels in MDA-MB-231 and MCF-7 cells were assayed as described under "Experimental Procedures." Values are the means \pm S.D. of three independent experiments performed in duplicate. ND indicates not detected.

Cell line and treatment	Polyamine levels in MDA-MB-231 cells					BENSp ^m
	Putrescine	Spermidine	Spermine	Acetylspermine	Acetylspermidine	
	nmol/mg protein					nmol/mg protein
231 cells, vector control	2.3 \pm 0.2	34.1 \pm 1.2	48.9 \pm 2.5	ND	ND	ND
231 cells, vector BENSp ^m	ND	8.7 \pm 0.7	12.1 \pm 1.1	0.7 \pm 0.1	4.4 \pm 0.7	38.6 \pm 3.7
231 cells, Δ SMO(PAOh1) control	2.8 \pm 0.3	32.5 \pm 3.4	54.3 \pm 2.4	ND	ND	ND
231 cells, Δ SMO(PAOh1) BENSp ^m	ND	8.9 \pm 0.9	13.9 \pm 2.9	1.9 \pm 0.3	8.8 \pm 0.5	36.4 \pm 2.1
231 cells, Δ SSAT control	1.8 \pm 0.3	38.6 \pm 1.4	39.8 \pm 2.5	ND	ND	ND
231 cells, Δ SSAT BENSp ^m	ND	20.6 \pm 0.5	19.1 \pm 0.8	ND	ND	41.3 \pm 4.9
231 cells, Δ SMO(PAOh1)/ Δ SSAT control	1.9 \pm 0.2	35.7 \pm 1.5	41.1 \pm 1.8	ND	ND	ND
231 cells, Δ SMO(PAOh1)/ Δ SSAT BENSp ^m	ND	20.1 \pm 0.4	26.4 \pm 0.5	ND	ND	38.5 \pm 3.1
Cell line and treatment	Polyamine levels in MCF-7 cells					BENSp ^m
	Putrescine	Spermidine	Spermine	Acetylspermine	Acetylspermidine	
	nmol/mg protein					nmol/mg protein
MCF-7 cells, vector control	3.7 \pm 0.1	68.3 \pm 5.6	50.3 \pm 4.4	ND	ND	ND
MCF-7 cells, vector BENSp ^m	1.3 \pm 0.2	7.5 \pm 0.6	5.1 \pm 0.5	8.7 \pm 0.6	15.9 \pm 0.7	47.1 \pm 2.5
MCF-7 cells, Δ SMO(PAOh1) control	3.8 \pm 1.0	60.1 \pm 6.6	59.2 \pm 5.6	ND	ND	ND
MCF-7 cells, Δ SMO(PAOh1) BENSp ^m	0.9 \pm 0.2	7.9 \pm 1.1	6.7 \pm 0.6	7.9 \pm 0.4	11.2 \pm 0.6	42.8 \pm 3.2
MCF-7 cells, Δ SSAT control	3.1 \pm 0.6	65.1 \pm 6.9	57.1 \pm 3.9	ND	ND	ND
MCF-7 cells, Δ SSAT BENSp ^m	1.9 \pm 0.1	52.4 \pm 5.2	40.9 \pm 2.5	ND	ND	46.2 \pm 1.8
MCF-7 cells, Δ SMO(PAOh1)/ Δ SSAT control	2.9 \pm 0.3	64.7 \pm 4.8	59.7 \pm 6.3	ND	ND	ND
MCF-7 cells, Δ SMO(PAOh1)/ Δ SSAT BENSp ^m	1.0 \pm 0.3	51.3 \pm 4.1	44.5 \pm 2.7	ND	ND	43.9 \pm 1.9

down of SSAT and SMO(PAOh1) on the polyamine content within each cell line. Knocking down SSAT, SMO(PAOh1), or the combination did not significantly alter the intracellular polyamine levels in untreated MDA-MB-231 or MCF-7 cells, suggesting that basal polyamine homeostasis in untreated breast cancer cell lines is not dependent on either SSAT or SMO(PAOh1) activity (TABLE TWO). BENSp^m treatment of MDA-MB-231 Δ SMO(PAOh1) and MCF-7 Δ SMO(PAOh1) cells reduced the intracellular polyamine levels to a similar extent (\sim 70–90%) as in BENSp^m-treated vector control cells or BENSp^m-treated wild-type parental cell lines (TABLE ONE). However, the knockdown of SSAT, either alone or in combination with SMO(PAOh1), in both MDA-MB-231 and MCF-7 cells reduced BENSp^m-induced polyamine depletion such that spermine and spermidine levels were only lowered by \sim 50% (TABLE TWO). Furthermore, although both acetylspermine and acetylspermidine were detected intracellularly in BENSp^m-treated vector and Δ SMO(PAOh1) cells, neither acetylated polyamine was detected in BENSp^m-treated Δ SSAT or Δ SSAT/ Δ SMO(PAOh1) cells. In addition, acetylspermine was detected in the media in BENSp^m-treated vector and Δ SMO(PAOh1) cells but not in the media from BENSp^m-treated Δ SSAT and Δ SSAT/ Δ SMO(PAOh1) MDA-MB-231 cells (data not shown). BENSp^m accumulation was similar in each of the cell lines examined. To determine whether the knockdown of SMO(PAOh1) or SSAT, either alone or in combination, affected other parts of the metabolic pathway, we chose to examine one of the rate-limiting steps in polyamine biosynthesis, ODC. The basal activity level of ODC was similar among all MDA-MB-231- and MCF-7-transfected cell lines (data not shown). ODC enzyme activity significantly decreased in all cell types regardless of SSAT or SMO(PAOh1) knockdown (ODC enzyme activity decreased significantly from 574.1 to 41.7 pmol of CO₂/mg of protein/h in BENSp^m-treated MDA-MB-231 vector-transfected cells and from 520.4 to 25.4 pmol/CO₂/mg of protein/h in BENSp^m-treated MDA-MB-231 Δ SSAT/ Δ SMO(PAOh1) cells ($p < 0.001$); ODC enzyme activity

decreased significantly from 1194.8 to 75.2 pmol CO₂/mg of protein/h in BENSp^m-treated MCF-7 vector-transfected cells and from 1221.8 to 61.8 pmol CO₂/mg of protein/h in BENSp^m-treated MCF-7 Δ SSAT/ Δ SMO(PAOh1) cells ($p < 0.001$).

SSAT, SMO(PAOh1), and SSAT/SMO(PAOh1) Knockdowns Differentially Reduce the Sensitivity of MDA-MB-231 Cells to BENSp^m Treatment—MTT assays were used to examine the effects of blocking SSAT and/or SMO(PAOh1) induction on the response of MDA-MB-231 and MCF-7 cells to BENSp^m treatment (Fig. 4). The knockdown of SMO(PAOh1) significantly reduced the sensitivity of MDA-MB-231 cells to BENSp^m with concentrations greater than 5 μ M BENSp^m but had no effect on the response of MCF-7 cells to BENSp^m. Co-treatment of MDA-MB-231 Δ SMO(PAOh1) cells with BENSp^m and MDL72527, the polyamine oxidase inhibitor, did not further alter their sensitivity to BENSp^m, providing further evidence that the induction of PAO does not play a role in their response to BENSp^m (data not shown). Knockdown of SSAT alone significantly reduced the sensitivity of MDA-MB-231 cells to BENSp^m with concentrations greater than 1 μ M and modestly reduced the sensitivity of MCF-7 cells to BENSp^m (Fig. 4). However, MDA-MB-231 Δ SSAT/ Δ SMO(PAOh1) cells were significantly less sensitive to BENSp^m than either of the single knockdown cells. As expected, the combined knockdown of these two enzymes in MCF-7 cells did not change their response to BENSp^m as compared with the knockdown of SSAT alone. Standard cell growth assays were performed to confirm these results using 1 and 10 μ M BENSp^m treatments for 96 h in each cell line, and similar results were obtained (data not shown). Flow cytometry analysis demonstrated that the knockdown of either SSAT or SMO(PAOh1) in MDA-MB-231 cells reduced the accumulation of cells in G₁ after BENSp^m treatment, although the knockdown of both enzymes nearly prevented the BENSp^m-induced G₁ block (data not shown). In MCF-7 cells, the knockdown of SSAT alone reduced the accumulation of cells in G₁, although dual knockdown did not further alter their response to BENSp^m (data not shown).

FIGURE 4. Effects of SMO(PAOh1) and SSAT knockdown on the sensitivity of breast cancer cell lines to BENSpM. Transfected MDA-MB-231 (A) and transfected MCF-7 (B) cells were exposed to increasing concentrations (0.1–25 μM) of BENSpM for 96 h. The effect on cell growth was assayed using the MTT assay as described under "Experimental Procedures." The results are the means \pm S.D. of three independent experiments performed in quadruplicate. Analysis of covariance demonstrated that for BENSpM $\geq 5 \mu M$, MDA-MB-231 Δ SMO(PAOh1) and MDA-MB-231 Δ SSAT cells were statistically less sensitive to BENSpM than MDA-MB-231 vector cells ($p = 0.020$ and $p = 0.005$, respectively). MDA-MB-231 Δ SSAT/ Δ SMO(PAOh1) cells were statistically less sensitive to BENSpM than either of the single knockdowns ($p < 0.001$ and $p < 0.001$). There was no statistically significant difference in the growth of cells between any of the MCF-7 cell lines.

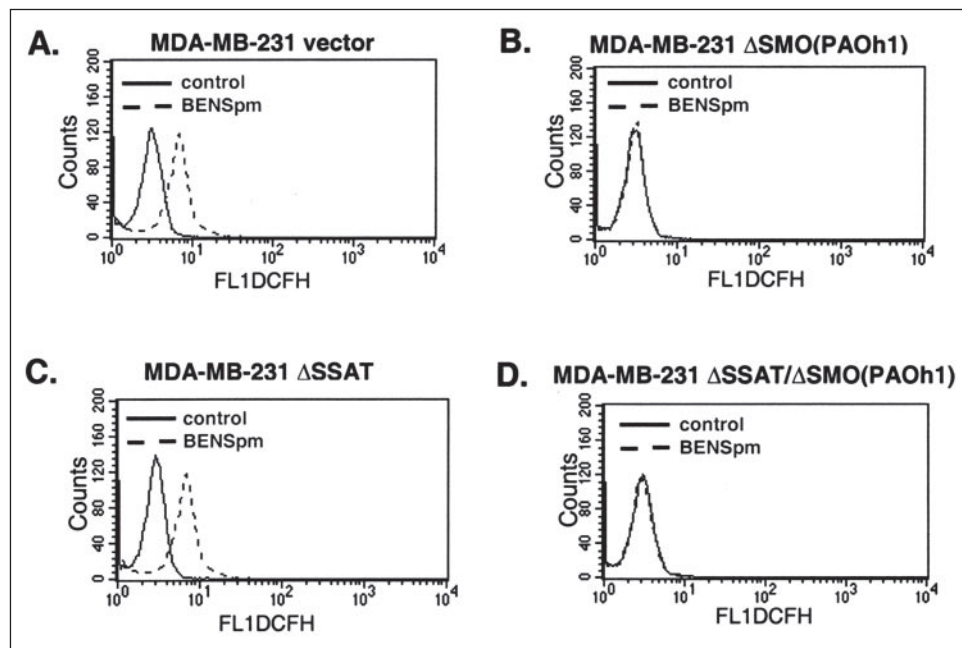
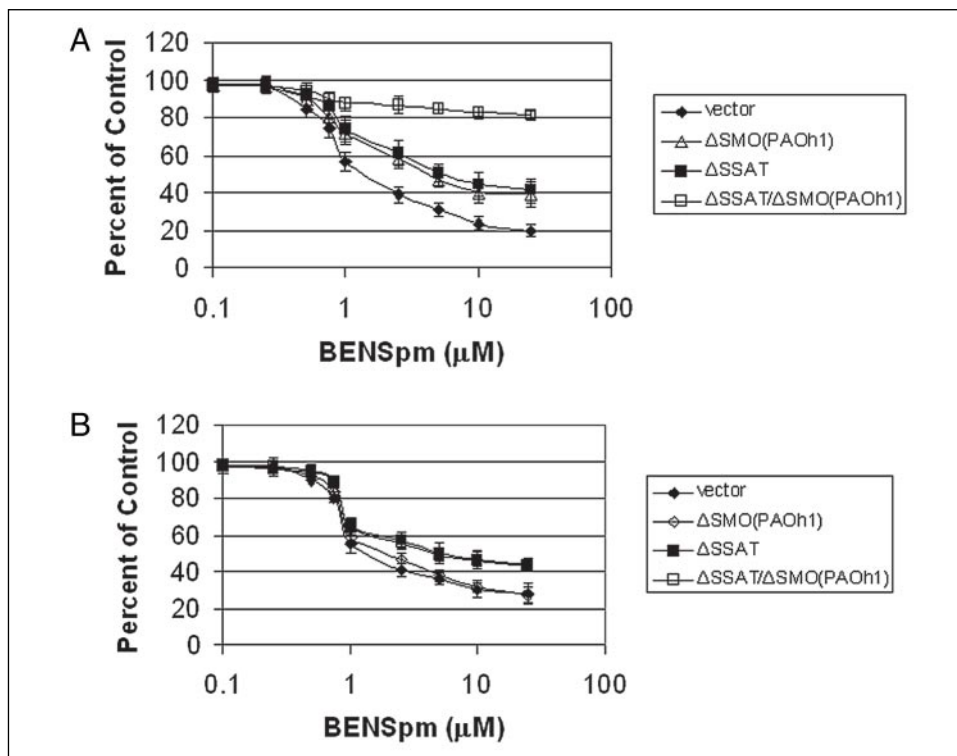


FIGURE 5. Effects of BENSpM-induced fluorescence in MDA-MB-231 cells detected by CM- H_2 DCFDA. MDA-MB-231 vector-transfected (A), MDA-MB-231 Δ SMO(PAOh1) (B), MDA-MB-231 Δ SSAT (C), and MDA-MB-231 Δ SSAT/ Δ SMO(PAOh1) (D) cells were treated with 10 μM BENSpM for 24 h, harvested, and treated with 10 μM CM- H_2 DCFDA for 30 min. 1×10^5 cells were analyzed by flow cytometry as described under "Experimental Procedures." The x axis represents F1 fluorescence intensity, and the y axis represents cell number. Shown are representative results from one of three experiments that gave similar results.

Effects of SSAT and SMO(PAOh1) Knockdown on Hydrogen Peroxide Production—To determine whether the production of hydrogen peroxide by polyamine catabolism plays a role in the antiproliferative effects of BENSpM and to determine which catabolic pathway is responsible for any H_2O_2 production, CM- H_2 DCFDA, an oxidation-sensitive fluorescent probe, was used to detect H_2O_2 production in BENSpM-treated MDA-MB-231 and MCF-7 cells. Treatment of MDA-MB-231 vector-transfected cells for 24 h with 10 μM BENSpM produced a significant increase in fluorescence comparable with that seen for treated wild-type cells (Fig. 5). However, co-treatment with either MDL72527, the polyamine oxidase inhibitor that inhibits both SMO(PAOh1) and PAO

enzyme activity, or catalase, which catalyzes the breakdown of H_2O_2 , prevented the increase in fluorescence over control (untreated) cells (data not shown). It is important to note that 25 μM MDL72527 was used to inhibit all oxidase activity in this study, significantly less than 250 μM , which was previously reported in other cell lines (22). To test the possibility that PAO was producing H_2O_2 but the H_2O_2 was rapidly detoxified by peroxisomal catalase, an inhibitor of catalase (AT) was used. Co-treatment of MDA-MB-231 cells with BENSpM and AT, or with BENSpM, AT, and catalase, still resulted in increased fluorescence, although co-treatment with BENSpM, AT, and MDL72527 did not increase fluorescence (data not shown). Furthermore, no change in flu-

SMO(PAOh1) Is the Primary Source of H₂O₂

orescence was seen in MDA-MB-231 Δ SMO(PAOh1) or MDA-MB-231 Δ SSAT/ Δ SMO(PAOh1) cells upon any treatment schedule. Exposure of MDA-MB-231 Δ SSAT cells to BENSpm increased fluorescence similar to BENSpm-treated MDA-MB-231 vector-transfected cells, indicating that SSAT induction by BENSpm does not lead to the production of H₂O₂ through PAO activity; rather the generation of H₂O₂ in BENSpm-treated MDA-MB-231 cells results primarily from the induction of SMO(PAOh1) activity. All of the controls examined were consistent with SMO(PAOh1) activity being the source of H₂O₂. No evidence of H₂O₂ production was seen in MCF-7 wild-type, vector, Δ SSAT, Δ SMO(PAOh1), or Δ SSAT/ Δ SMO(PAOh1) cells with BENSpm (data not shown).

DISCUSSION

Induction of polyamine catabolic enzymes has been studied as a potential strategy for anti-cancer therapy (7, 9, 15, 16). The recent cloning of SMO(PAOh1) has enhanced our ability to examine the role that polyamine catabolism may play in cancer (18). SMO(PAOh1) oxidizes spermine to spermidine and produces the toxic reactive oxygen species, H₂O₂. However, the reactive oxygen species production through induced polyamine catabolism had been hypothesized previously to occur through the classical two-step catalysis by SSAT/PAO (15–17). Reactive oxygen species are known to contribute to a number of destructive processes within cells, including DNA damage, and have been implicated in the cytotoxic effects of some polyamine analogues (11, 14, 16, 34, 35). It was hypothesized that the H₂O₂ produced by polyamine catabolism contributes to cell growth inhibition, and pharmacologic manipulation of its activity could be used as a strategy to target tumor cells. However, the source or sources of H₂O₂ and the exact role that each polyamine catabolic enzyme plays in the response to polyamine analogue exposure have not been conclusively determined.

The results presented here demonstrate that RNA interference can be used effectively to knock down the mRNA expression and enzyme activity of two key polyamine catabolic enzymes in human breast cancer cell lines and report the first use of stable siRNAs directed against multiple polyamine metabolic enzymes simultaneously. The stable knockdown of SSAT in this study prevented BENSpm-induced SSAT activity by ~95% and also reduced the BENSpm-induced depletion of polyamine levels in both cell lines. These results are consistent with studies from Chen *et al.* (28) who reported the use of transient siRNA suppression of SSAT in SK-MEL-28 human melanoma cells. By using a transient knockdown approach, they demonstrated the role of SSAT in spermine depletion and caspase-mediated apoptosis. However, the stable nature of the cell lines generated here provides a more useful system for the examination of long term effects than that afforded by the transient knockdown system reported previously.

The data presented here also demonstrate that SMO(PAOh1) mRNA and activity are differentially induced by BENSpm in several breast cancer cell lines. No induction of PAO mRNA or activity was seen in any of the BENSpm-treated breast cancer cell lines examined (Fig. 1), suggesting that in the breast cancer cell lines examined, PAO is constitutively expressed at low levels. In addition, no significant expression or induction by BENSpm of SMO(PAOh1) was seen in an immortalized nontumorigenic mammary epithelial cell line, MCF-10A (data not shown). The BENSpm-induced oxidase activity utilized only spermine, the preferred SMO(PAOh1) substrate, as a substrate in MDA-MB-231 and Hs578t cells and showed no activity with either N¹-acetylspermine, the preferred PAO substrate, or spermidine (data not shown). Vujcic *et al.* (19) recently reported that BENSpm can induce both PAO and SMO(PAOh1) mRNA in HEK-293 cells. However, the real time PCR

and enzyme activity data shown here suggest that PAO is not induced by BENSpm treatment in these breast cancer cell lines.

A key question was whether the antiproliferative effects of BENSpm are due, in part, to the induction of either of the polyamine catabolic pathways and the associated H₂O₂ production. BENSpm treatment of MDA-MB-231 vector cells resulted in increased fluorescence (detected using the oxidation-sensitive fluorescent probe CM-H₂DCFDA), indicative of an increase in reactive oxygen species. Co-treatment of these cells with MDL72527 or catalase prevented the increase in fluorescence, and furthermore, the specific knockdown of SMO(PAOh1) prevented the BENSpm-induced shift in fluorescence. Because the siRNA targeting SSAT had no effect on measurable H₂O₂, the H₂O₂ produced in BENSpm-exposed MDA-MB-231 cells must originate from SMO(PAOh1) activity induction, not from PAO activity as proposed previously (11). Furthermore, results obtained using AT demonstrate that inhibition of catalase activity does not alter the FACS profile of either untreated or BENSpm-treated cells, indicating that endogenous catalase is not involved in detoxifying PAO-produced H₂O₂ and confirming that the primary enzyme involved in the production of H₂O₂ in response to BENSpm in the human breast cancer cell lines examined is SMO(PAOh1).

It was initially hypothesized that the induction of SSAT enzyme activity contributed to the antiproliferative effects of BENSpm through production of the substrate for PAO activity, the acetylated polyamines, and that the cell growth inhibition resulted from PAO-produced H₂O₂. However, no PAO enzyme activity was detected in any BENSpm exposed cell line, and furthermore, no H₂O₂ was detected in MDA-MB-231 Δ SMO(PAOh1) cells. After polyamines are acetylated by SSAT, they can either be oxidized by PAO or be exported from the cell (1–3). Because no PAO activity was observed, the induction of SSAT was hypothesized to acetylate the polyamines, which were then exported from the cell. Therefore, the main effect of SSAT induction was the reduction of intracellular polyamine levels. The knockdown of SMO(PAOh1) in MDA-MB-231 and MCF-7 cells had no significant effect on BENSpm-induced reduction in intracellular polyamine levels. However, BENSpm treatment of SSAT knockdown cells resulted in a smaller reduction of intracellular polyamine levels as did BENSpm treatment of vector or Δ SMO(PAOh1) cells. Furthermore, although the acetylated polyamines were detected intracellularly and in the media of BENSpm-treated vector and Δ SMO(PAOh1) MDA-MB-231 and MCF-7 cells, neither of the acetylated polyamines were detected intracellularly or in the media from either of the SSAT knockdown cells. These results indicate that BENSpm-induced SSAT enzyme activity results in the acetylation of spermine and spermidine, which are subsequently exported from the cell rather than serving as a substrate for PAO, thus resulting in decreased intracellular polyamine levels. The down-regulation of ODC enzyme activity frequently coincides with cell growth inhibition and is likely involved in the antiproliferative effects of BENSpm found in this study (36–38). More importantly, the knockdown of SSAT and/or SMO(PAOh1) did not affect the down-regulation of ODC enzyme activity by BENSpm treatment in MDA-MB-231 or MCF-7 cells, suggesting that other parts of the polyamine metabolic pathway are not affected in the knockdown cell lines.

The results of this study provide further evidence that the effects of polyamine analogues are cell line-specific. In MDA-MB-231 cells, blocking the induction of either SMO(PAOh1) or SSAT individually reduces the sensitivity to BENSpm. When the induction of both SSAT and SMO(PAOh1) is prevented, MDA-MB-231 cells become significantly more resistant to the growth inhibitory effects of BENSpm than the vector controls or the individual knockdowns alone. These results

demonstrate that the antiproliferative effects of BENSpm can be mediated through both SSAT and SMO(PAOH1) induction. Thus, agents that target the induction of both of these catabolic enzymes may possess greater antiproliferative activity as compared with agents that target only one of the enzymes. Targeting specific tumor types with agents that induce both SSAT and SMO(PAOH1) may be a rational approach to improve the design of antitumor polyamine analogues.

In summary, this study demonstrates that SMO(PAOH1) and SSAT are major targets of BENSpm in specific human breast cancer cell lines and, furthermore, that these catabolic enzymes act together in determining the response of some breast cancer cell lines to BENSpm. The antiproliferative effects of BENSpm in MDA-MB-231 cells are mediated in part through the production of H₂O₂ by SMO(PAOH1) and by the export of acetylated polyamines formed by the activity of SSAT. These results demonstrate the independent effects of the polyamine catabolic enzymes in response to polyamine analogue treatment and provide insight for the development of more specific anticancer agents for the treatment of breast cancer.

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Polyamine Analogues Down-regulate Estrogen Receptor α Expression in Human Breast Cancer Cells*

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Yi Huang[‡], Judith C. Keen^{‡1}, Allison Pledgie[‡], Laurence J. Marton[§], Tao Zhu[‡], Saraswati Sukumar[‡], Ben Ho Park[‡], Brian Blair[‡], Keith Brenner[‡], Robert A. Casero, Jr.[‡], and Nancy E. Davidson^{‡2}

From the [‡]Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, Maryland 21231 and [§]Cellgate Inc., Redwood City, California 94065

The critical role of polyamines in cell growth has led to the development of a number of agents that interfere with polyamine metabolism including a novel class of polyamine analogues, oligoamines. Here we demonstrate that oligoamines specifically suppress the mRNA and protein expression of estrogen receptor α (ER α) and ER α target genes in ER-positive human breast cancer cell lines, whereas neither ER β nor other steroid hormonal receptors are affected by oligoamines. The constitutive expression of a cytomegalovirus promoter-driven exogenous ER α in ER-negative MDA-MB-231 human breast cancer cells was not altered by oligoamines, suggesting that oligoamines specifically suppress ER α transcription rather than affect mRNA or protein stability. Further analysis demonstrated that oligoamines disrupted the DNA binding activity of Sp1 transcription factor family members to an ER α minimal promoter element containing GC/CA-rich boxes. Treatment of MDA-MB-231 cells with the JNK-specific inhibitor SP600125 or expression of the c-Jun dominant negative inhibitor TAM67 blocked the oligoamine-activated JNK/c-Jun pathway and enhanced oligoamine-inhibited ER α expression, suggesting that AP-1 is a positive regulator of ER α expression and that oligoamine-activated JNK/AP-1 activity may antagonize the down-regulation of ER α induced by oligoamines. Taken together, these results suggest a novel antiestrogenic mechanism for specific polyamine analogues in human breast cancer cells.

Polyamines are naturally occurring polycationic alkylamines that are absolutely required for cell growth. Because of their positively charged amine groups, polyamines interact with negatively charged molecules like DNA, RNA, proteins, and phospholipids to modify their structure and conformation. Rapid tumor growth is associated with significantly increased poly-

amine biosynthesis (1, 2). In human breast cancer, studies have demonstrated the important roles of polyamine biosynthesis and action in tumor development and metastasis, and increased polyamine levels are often associated with aggressive forms of breast tumors (3–7). Because of the requirement for polyamines in breast cell growth and the demonstration of dysregulated polyamine metabolism in breast tumor cells, polyamine metabolism has become a rational target for breast cancer therapy. Polyamine analogues were designed based upon the theory that the analogues can mimic some of the self-regulatory functions of natural polyamines but are unable to substitute for natural polyamines in their growth promoting roles (8). Recently, a novel class of polyamine analogues has been developed that includes conformationally restricted, cyclic, and long chain oligoamine analogues (9). Our recent studies showed that oligoamines effectively inhibit growth of human breast cancer cell lines in culture and mouse xenografts (10). We also demonstrated that specific oligoamines reduced ornithine decarboxylase activity and induced the activity of the polyamine catabolic enzyme, spermidine/spermine N^1 -acetyltransferase, thereby significantly decreasing the intracellular polyamine pools in several human breast cancer cell lines (10, 11).

Estrogens are thought to play a major role in breast cancer development. Because of the pivotal role of the estrogen-estrogen receptor (ER)³ axis in breast cancer progression, targeting ER or its ligands is a major strategy for breast cancer treatment. Estrogen effects are exerted through the activation of specific estrogen receptors. Unfortunately, many breast tumors develop estrogen independence, perhaps as a consequence of multiple mechanisms including altered balance of co-regulatory proteins or the emergence of other growth signaling pathways. These molecular mechanisms are still poorly understood, and this lack of understanding has posed a major clinical challenge in breast cancer therapy. Therefore, elucidation of ER regulatory pathways is critical. A potential interaction between polyamines and ER is suggested by several lines of evidence. For instance, in ER-positive breast cancer cells, estradiol up-regulates ornithine decarboxylase and increases polyamine levels, which accompanies cell proliferation (12). Tamoxifen-induced growth inhibition is often associated with the down-regulation of polyamine biosynthesis (13). It also has been demonstrated that spermine facilitates the binding of ER α to estrogen response elements (ERE) and that increased polyamine

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¹ Present address: Coriell Institute for Medical Research, Camden, New Jersey 08106.

² To whom correspondence should be addressed: Breast Cancer Program, The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, 1650 Orleans St., Rm. 409, Baltimore, MD 21231. Tel.: 410-955-8489; Fax: 410-614-4073; E-mail: davidna@jhmi.edu.

³ The abbreviations used are: ER, estrogen receptor; ERE, estrogen response element; PR, progesterone receptor; JNK, c-Jun NH₂-terminal kinase; CMV, cytomegalovirus; EMSA, electrophoretic mobility shift assay.

concentrations may induce a DNA sequence containing EREs to undergo conformational transition and enhance the binding of ER to EREs, suggesting a possible mechanism for polyamine-mediated alterations in the interaction of ER with ERE (14).

In this study, we demonstrate that selective oligoamines specifically suppress the expression and activity of ER α in human breast cancer cells. Oligoamines disrupt the DNA binding activity of Sp1 transcription factor family members at the GC- and CA-rich boxes of the ER α proximal promoter. Additionally, a c-Jun NH₂-terminal kinase (JNK) specific inhibitor or expression of a dominant negative c-Jun inhibited ER α expression and enhanced inhibition of ER α expression by the oligoamines, suggesting that the JNK/AP-1 signaling pathway is a positive regulator of ER α expression and that oligoamine activated c-Jun activity may antagonize the down-regulation of ER α by oligoamines.

EXPERIMENTAL PROCEDURES

Compounds and Culture Conditions—Oligoamines were synthesized as previously reported (9, 15). Stock solutions (10 mM in double distilled H₂O) of each analogue were diluted with medium to the desired concentrations for specific experiments. Spermine was purchased from Sigma and was prepared as a stock solution of 10 mM in H₂O. 17 β -Estradiol was purchased from Sigma. SP600125 (anthra[1,9-cd]pyrazol-6(2H)-one) from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA) was prepared as a stock solution of 20 mM in 100% Me₂SO. The human breast cancer MCF-7, T47D, and MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 2 mM glutamine at 37 °C in a 5% CO₂ atmosphere.

Plasmid Construction and Stable Transfection—The entire coding region of ER α was PCR-amplified using MCF7 cDNA as template and cloned into the expression vector, pIRESNEO (Clontech, Palo Alto, CA). The cloned cDNA was verified by sequencing (16). Empty vector or pIRES-ER construct was then transfected into MDA-MB-231 cells using Gene Jammer as recommended by the manufacturer (Invitrogen). Stable colonies were selected and maintained using G418 at a concentration of 500 μ g/ml in Dulbecco's modified Eagle's medium. The cDNA of mutant c-Jun (TAM67), which lacks amino acids 3–122 of the transactivation domain, was a generous gift from Drs. Steve Georas (Johns Hopkins) and Michael Birrer (National Institutes of Health) and was subcloned into the pcDNA3.1 mammalian expression vector (Invitrogen) (11). TAM67 constructs were transfected into MCF-7 cells using the same method described above, and stable transfectants were selected and maintained as above.

Immunoblotting—Whole cellular proteins were extracted as previously described (10). Cytoplasmic and nuclear fractions were prepared using the NE-PERTM nuclear and cytoplasmic extraction kit (Pierce). The protein concentrations were determined, and Western blotting was performed using the methods described previously (10). Primary antibodies against ER α , ER β , vitamin D receptor, progesterone receptor (PR), retinoic acid receptor β , cyclin D1, Sp1, Sp3, c-Jun, and JNK1 were from Santa Cruz Biotechnology Company (Santa Cruz, CA). Proliferating cell nuclear antigen monoclonal antibody was pur-

chased from Oncogene Research Products (Cambridge, MA). Actin was used to normalize for protein loading. All of the experiments were performed at least twice with similar results.

Luciferase Reporter Activity Assays—Deletion constructs of the ER α 5'-flanking region in luciferase reporter vector pGL3-Basic were provided by Dr. Suzanne Fuqua (Baylor College of Medicine). ERE-tk-luc vectors contain four consecutive ERE and were used to measure ER-mediated transcription activity. The cells were seeded at 2×10^5 cells/well in 24-well plates 24 h prior to transfection. 3 μ l of Gene Jammer transfection reagent (Stratagene, La Jolla, CA) was used to transiently transfect 0.5 μ g of luciferase reporter gene constructs. One μ g of β -galactosidase expression vector was co-transfected with all plasmids to determine transfection efficiency. Luciferase activity was measured on a Monolight luminometer using the BrightGlo luciferase assay kit (Promega, Madison, WI), and β -galactosidase activity was determined using the β -galactosidase activity kit (Promega). The experiments were completed three times in triplicate. The Student's *t* test was used to determine the statistical differences between various experimental and control groups. *p* \leq 0.05 was considered significant. Shown are means \pm S.D.

Reverse Transcription PCR—RNA was harvested using TRIzol reagent (Invitrogen) as previously described (16). cDNA was synthesized from 3 μ g of total RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen) and oligo(dT) primers (Invitrogen) at 37 °C for 1 h. PCR was performed using the following primers: ER α sense, GCA CCC TGA AGT CTC TGG AA; ER α antisense, TGG CTA AAG TGG TGC ATG AT (55 °C for 35 cycles); actin sense, ACC ATG GAT GAT GAT ATC GC; and actin antisense, ACA TGG CTG GGG TGT TGA AG (60 °C for 30 cycles).

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear and cytoplasmic proteins were extracted using the NE-PERTM nuclear and cytoplasmic extraction kit (Pierce). EMSA was performed as described previously (16, 17). Briefly, 10 μ g of nuclear extracts were incubated with γ -³²P-labeled oligonucleotides (2×10^4 counts/min/lane), which span the ER α promoter from –245 to –182 bp. For supershift assays, the cell extracts were preincubated with 2 μ g of antibody for 20 min on ice. A 100-fold excess of unlabeled 21-bp oligonucleotide probe, which contains the minimal consensus binding site for Sp1 transcription factor (Santa Cruz Biotechnology Company) was used as a competitor. The reactions were then separated on 5% polyacrylamide gels at 150 V in 0.5 \times Tris borate-EDTA buffer followed by autoradiography.

Chromatin Immunoprecipitation—Chromatin immunoprecipitation assays were performed as described previously (18). After treatment, the cells were exposed to formaldehyde to cross-link proteins, and chromatin samples were sonicated on ice three times for 10 s each (to give an average length of 1–1.5 kb sheered genomic DNA) followed by centrifugation for 10 min. The antibodies for Sp1 family members were used for immunoprecipitation of protein-DNA complexes. PCR primers flanking the Sp1-binding sites of the ER α promoter were designed as follows: sense, 5'-ACC TTA GCA GAT CCT CGT; and antisense, 5'-GCT GCT GGA TAG AGG CTG A. Genomic DNA was used as a positive control (input). Chromatin eluted

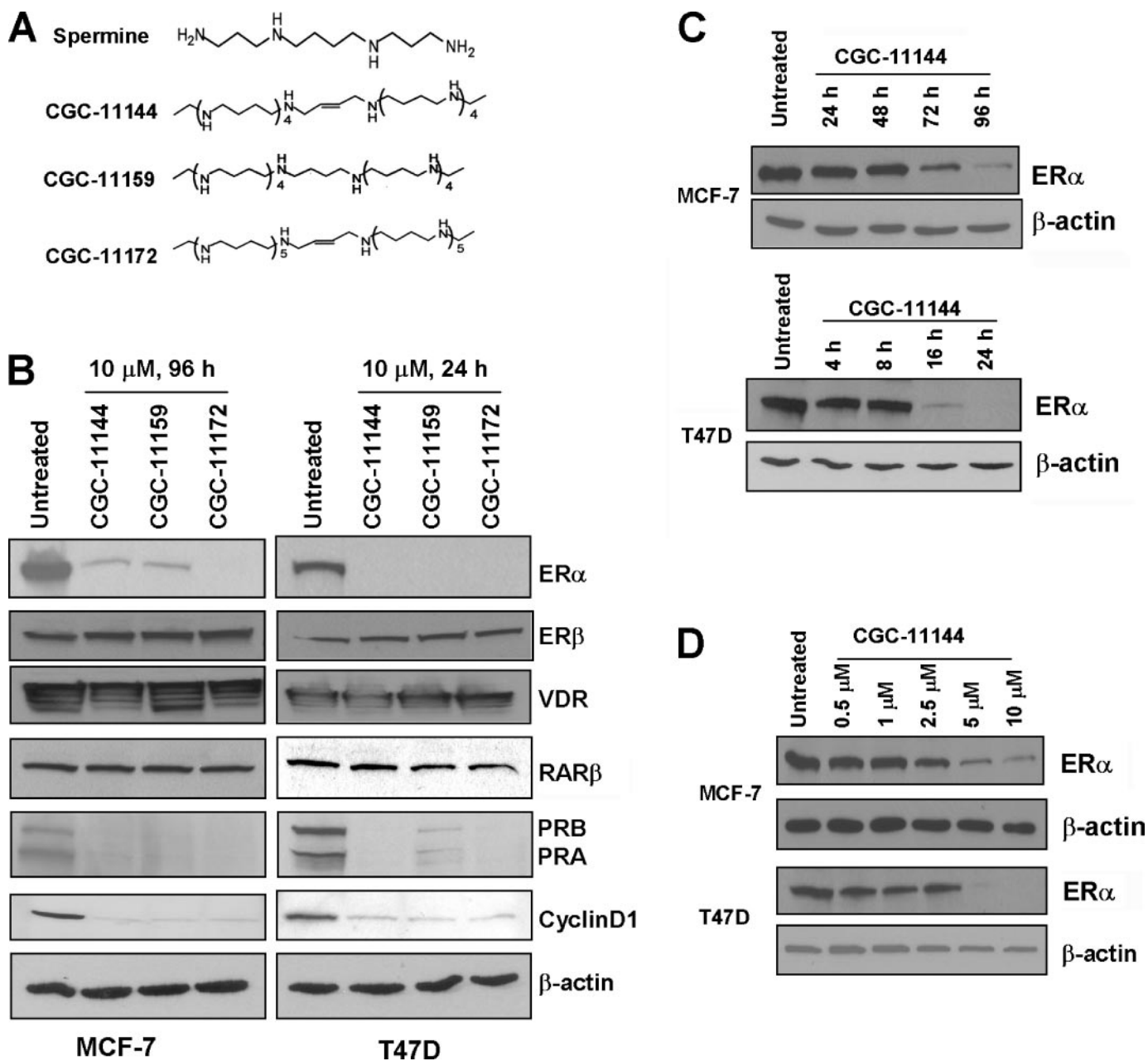


FIGURE 1. Effects of oligoamines on ER α expression. *A*, structures of natural spermine and oligoamines. *B*, MCF-7 and T47D cells were treated with 10 μ M of the indicated oligoamines for 96 and 24 h, respectively. Western blots were performed to detect the expression of ER α , ER β , vitamin D receptor, PRA, PRB, and cyclin D1. *C*, MCF-7 and T47D cells were treated with 10 μ M CGC-11144 for the indicated times. Western blots were performed to detect the expression of ER α . *D*, MCF-7 and T47D cells were treated with increasing concentrations of CGC-11144 for 96 h in MCF-7 and 24 h in T47D, respectively. Western blots were performed to detect the expression of ER α . Actin protein was blotted as a control. All of the experiments were performed at least twice with similar results.

from immunoprecipitations lacking antibody was used as a “no antibody” control. PCR products were run on 1% agarose gel and analyzed by ethidium bromide staining.

Immunoprecipitation and Kinase Assays—The immunoprecipitation of JNK kinase and kinase activity assays were performed as described previously (19). Briefly, JNK complexes were immunoprecipitated by anti-JNK rabbit polyclonal antibodies (Santa Cruz Biotechnology) bound to protein A-Sepharose (Amersham Biosciences). The kinase assays were performed by the addition of 2 μ g of substrate protein, 20 μ M cold ATP, and 10 μ Ci of [γ - 32 P] ATP for 30 min at 30 $^{\circ}$ C and stopped by the addition of 2 \times SDS-PAGE sample buffer. The product

was analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. The experiments were performed at least twice with similar results.

RESULTS

Inhibition of ER α Expression by Oligoamines—Three representative oligoamine analogues, CGC-11144, CGC-11159, and CGC-11172, were selected for this study (Fig. 1*A*). To investigate whether oligoamines affect hormonal signaling pathways in breast cancer cells, the protein expression of several breast cell growth-related hormone receptors including the ER α , ER β , vitamin D receptor, and retinoic acid receptor β were exam-

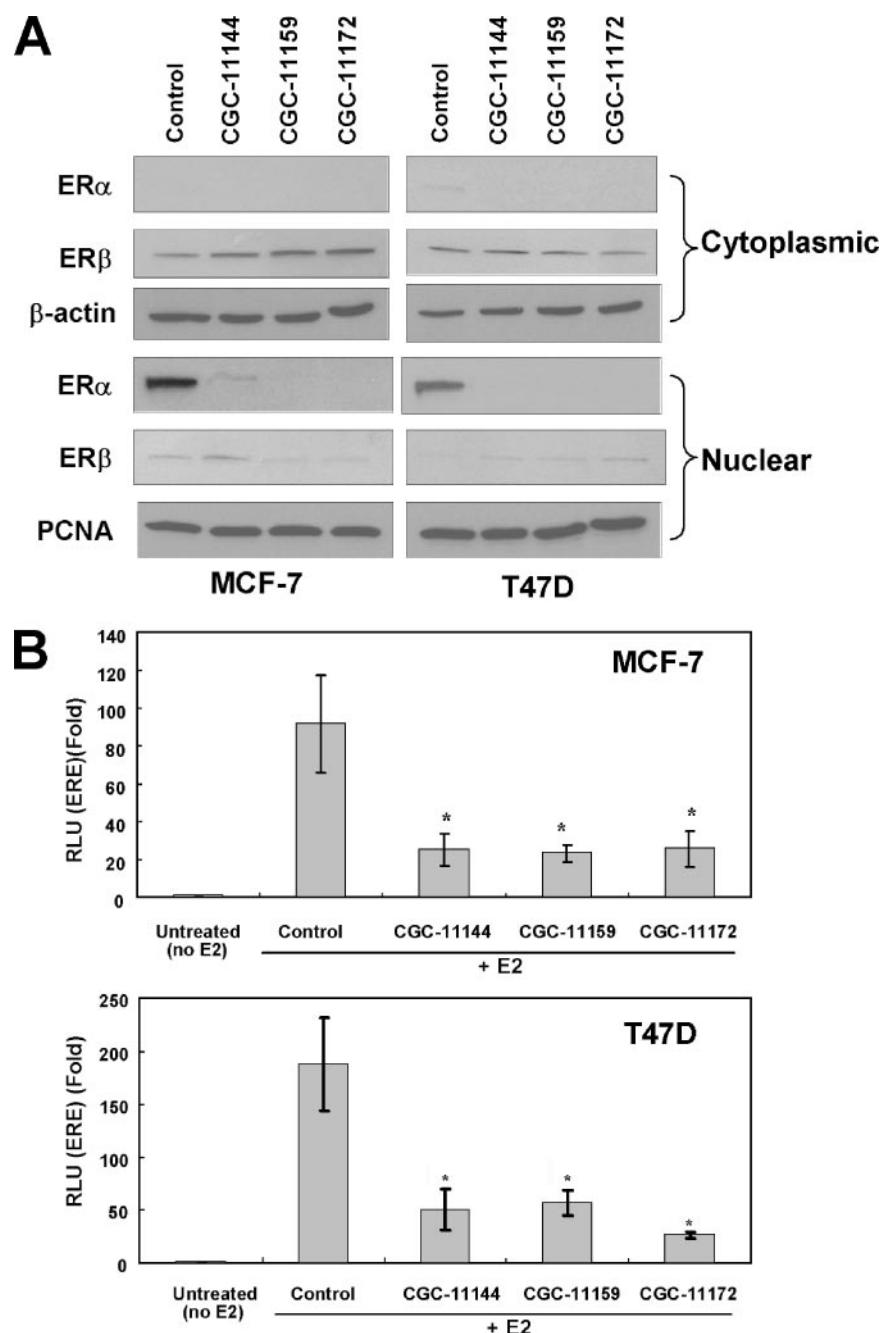


FIGURE 2. Effects of oligoamines on transactivation of ER α . The cells were cultured in phenol red-free Dulbecco's modified Eagle's medium supplemented with 5% charcoal dextran-stripped fetal bovine serum and treated with 10 nM 17 β -estradiol (E₂). *A*, MCF-7 and T47D cells were treated with 10 μ M of the specific oligoamine for 96 h in MCF-7 and 24 h in T47D cells. Cytoplasmic or nuclear protein was extracted for immunoblotting with anti-ER α and ER β antibodies. Actin protein was blotted as a loading control for cytoplasmic extracts and proliferating cell nuclear antigen (PCNA) was used as a loading control for nuclear extracts. *B*, MCF-7 and T47D cells were co-transfected with ERE-tk-luciferase and CMV- β -galactosidase. Reporter gene activities were measured after 24 h of oligoamine treatment for T47D cells and 96 h treatment for MCF-7 cells. The experiments were completed three times, and each measurement was taken in triplicate. *, $p < 0.05$, statistically significant differences using Student's t test between control cells (treated with E₂ only) and cells treated with E₂ and oligoamines.

ined. All three oligoamines specifically down-regulated the protein level of ER α in two ER-positive human breast cancer cell lines MCF-7 and T47D, whereas the protein expression of ER β , vitamin D receptor, and retinoic acid receptor β was not affected by oligoamines (Fig. 1B). Further, Western blot analysis showed that the expression of two ER α -regulated genes,

transiently transfected with the ERE-tk-luciferase constructs. Reporter gene activities were measured after 24 h of oligoamine treatment in T47D cells and after 96 h of treatment in MCF-7 cells. E₂ significantly increased ERE reporter gene activities in both ER-positive human breast cancer cell lines. Treatment with all oligoamines significantly suppressed the level of

progesterone receptors (PRA and PRB), and cyclin D1 was also inhibited by oligoamines (Fig. 1B). These data suggest that oligoamines specifically target ER α and its downstream genes in breast cancer cells. Down-regulation of ER α by oligoamines displays clear time-, concentration-, and cell type-dependent features in that ER α expression was inhibited by oligoamines after a 72-h exposure in MCF-7 cells and a 16-h exposure in T47D cells (Fig. 1C). A minimum CGC-11144 concentration of 5 μ M is required to decrease ER α protein in MCF-7 cells treated for 96 h and T47D cells treated for 24 h (Fig. 1, C and D).

Oligoamines Down-regulate ER-mediated Transcription Activity—Ligand-activated ER dimers can bind to the estrogen response element (ERE) of target genes and regulate their transcription. To determine whether oligoamine down-regulation of ER α affects ER-mediated transcriptional activity, MCF-7 or T47D cells were treated with various oligoamines for different times with 10 nM 17 β -estradiol (E₂), and the subcellular localization of ER α and ER β was examined by immunoblotting using nuclear and cytoplasmic extracts. Treatment with E₂ alone (control) led to nuclear localization of ER α , whereas the nuclear ER α protein was significantly decreased by treatment with oligoamines. By contrast, most of the ER β protein remained in the cytosol after the stimulation of E₂, and oligoamine treatment did not affect either the level of protein expression or the cellular localization of ER β (Fig. 2A). Polyamine analogues specifically down-regulate ER α expression without affecting the expression of ER β . To verify that decreased ER α expression is associated with decreased ER α -mediated transcription activities, MCF-7 and T47D cells were

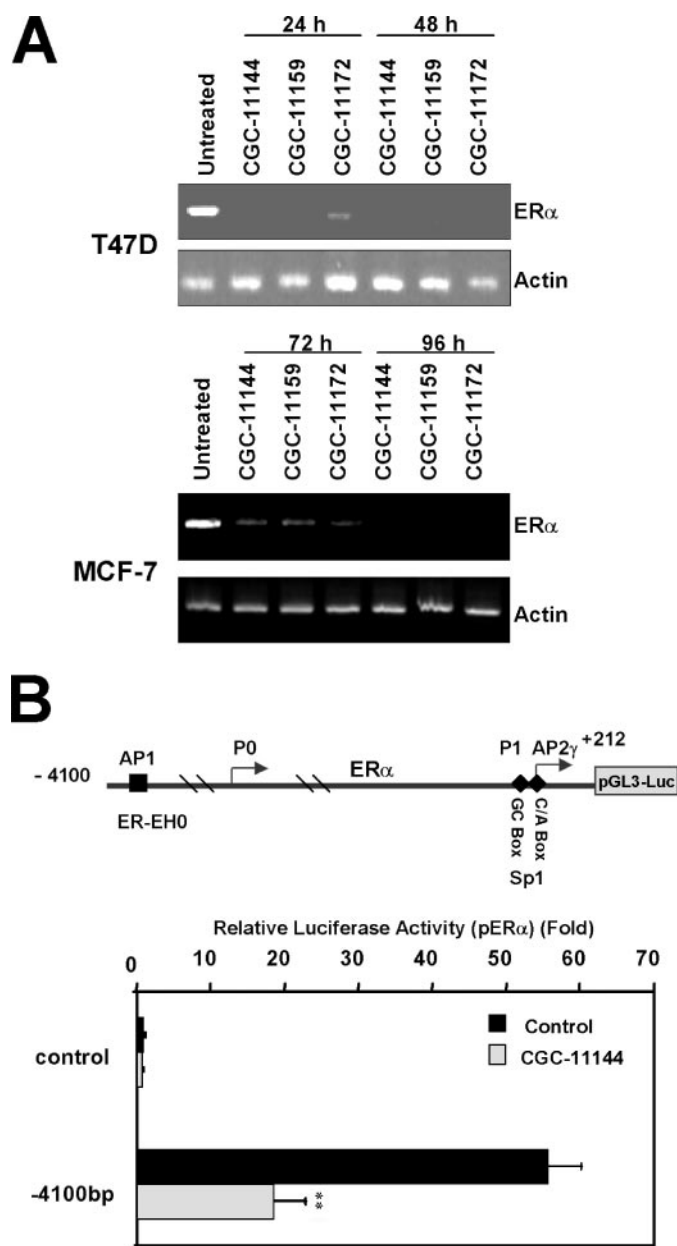


FIGURE 3. Effects of oligoamines on ER α mRNA expression and promoter activity. A, MCF-7 and T47D cells were treated with the specific oligoamine for the time indicated. ER α mRNA expression was determined by reverse transcription-PCR. B, luciferase reporter constructs containing the ER α promoter region from -4100 to +212 bp were transfected with CMV- β -galactosidase into T47D cells followed by treatment of 10 μ M CGC-11144 for 24 h. Reporter gene activities were then measured. The experiments were completed three times, and each measurement was taken in triplicate. **, $p < 0.01$.

reporter gene activity in both cell lines, suggesting that down-regulation of ER α protein expression by oligoamines leads to the subsequent suppression of ER-mediated transcriptional activities (Fig. 2B).

Down-regulation of ER α by Oligoamines Is Caused by Transcriptional Repression—To elucidate the possible mechanism by which oligoamines down-regulate ER α , reverse transcription-PCR was performed to determine the ER α mRNA level in MCF-7 and T47D cells after treatment with oligoamines. Reverse transcription-PCR results showed that ER α mRNA was

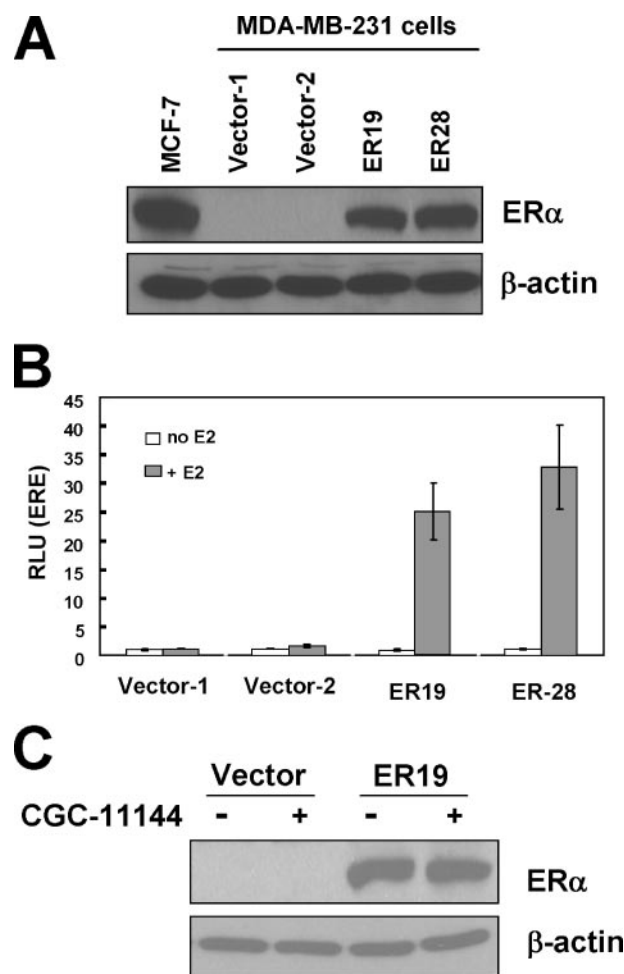


FIGURE 4. Effects of oligoamines on exogenous ER α expression in MDA-MB-231 cells. A, MDA-MB-231 cells were stably transfected with pIRES-ER α . Proteins isolated from MCF-7 cells, empty vector MDA-MB-231 transfectants, and ER α transfected MDA-MB-231 single colonies were subjected to immunoblot with an ER α antibody. Two G418-resistant clones (ER19 and ER28) were shown to express high levels of ER α . B, vector control and ER α transfected MDA-MB-231 cells were co-transfected with ERE-tk-luciferase and CMV- β -galactosidase and cultured in medium with 5% charcoal dextran-stripped fetal bovine serum with or without 10 nM E $_2$. The bars represent the means \pm S.D. of at least three independent experiments. C, vector control and ER α transfected ER19 cells were treated with oligoamines for 72 h, and ER α expression was determined by Western blot.

markedly decreased by oligoamines in both cell lines (Fig. 3A). To investigate whether the down-regulation of ER α by oligoamines occurs through the regulation of the promoter activity of the ER α gene, 4.1 kb of the ER α 5'-flanking region linked to a luciferase reporter pGL3-Basic vector was transiently transfected into T47D cells followed by treatment with CGC-11144 as a representative oligoamine. The results showed that endogenous ERE-luciferase activity is significantly suppressed by CGC-11144 (Fig. 3B).

To further validate that oligoamine-induced down-regulation of ER α is caused by the repression of ER transcription rather than by enhanced protein degradation, the ER-negative MDA-MB-231 cell line was stably transfected with an ER α cDNA construct that lacks any 5' ER α promoter sequence and that is driven by the CMV promoter in a pIRES vector (MDA-MB-231-ER α). Two single clones (ER19 and ER28) that express ER α at a level comparable with that observed in MCF-7 cells

were identified and selected for a series of functional assays (Fig. 4A). To test whether constitutively expressed ER α is functional in MDA-MB-231 cells, an ERE-tk-luciferase reporter gene activity assay was used to demonstrate that E₂ induced ERE reporter gene expression (luciferase) in both ER α transfected clones, but not in vector controls (Fig. 4B). These results indicate that exogenously introduced ER α in ER α -negative breast cancer cells has intact ER signaling in response to estradiol. Further, Western blotting demonstrated that the ER α protein level was not affected by oligoamine treatment in either clone ER19 (Fig. 4C) or ER28 (not shown), thereby providing evidence that oligoamine-induced down-regulation of ER α indeed occurs through repression of ER α promoter activity rather than enhanced protein degradation.

Oligoamines Disrupt ER α Promoter Activity within a Proximal Region Containing an Sp1-binding Site—The exact molecular mechanisms for regulation of ER α expression in breast cancer cells are unclear, but studies suggest that the regulation is at least partially transcriptional (20). Using deletion luciferase reporter constructs of the ER α promoter, we found that CGC-11144 inhibited the reporter gene activity of the proximal ER α minimal promoter region -245 to $+212$ bp (Fig. 5A). This fragment contains the GC- and CA-rich boxes that are binding sites for Sp1 transcription factor family members and other zinc finger transcription factors. To ascertain whether oligoamines alter the recruitment of Sp1 family members to this element, a radiolabeled oligonucleotide spanning the ER α promoter region from -245 to -182 bp was used as a probe with T47D nuclear extracts in EMSA analysis. Shifted protein-DNA complexes were clearly observed in untreated cells, and oligoamines markedly reduced protein binding to this element (Fig. 5B). The binding of Sp1 to this element was confirmed by supershift analysis (Fig. 5B). Competition with an unlabeled probe consisting of the minimal Sp1 consensus sequence clearly inhibits the binding of transcription factor complex to the ER α minimal promoter region (Fig. 5C). This result suggests that the EMSA is generated by a protein contacting the Sp1-binding site at the ER α minimal promoter and not other sequences, such as the E box. In addition, the labeled probe was incubated with Sp1 antibody only, and no shift was observed. This result rules out the possibility of a direct interaction between labeled probe and antibody (Fig. 5C).

To further investigate the mechanisms underlying the interruption of the DNA binding activity of the Sp1 family of transcription factors by oligoamines, Western analysis was used to examine whether oligoamines affected the protein expression of Sp1 and/or Sp3, two transcription factors that directly bind to GC boxes and transactivate the ER minimal promoter (17). CGC-11144 did not change the protein level of Sp1 or Sp3 (Fig. 5D), indicating that its interruption of Sp1 transcription factor family activity is not through diminished protein expression. Using the chromatin immunoprecipitation assay, the binding of Sp1 and Sp3 to the -245 to -182 element of the ER α promoter was confirmed, and CGC-11144 treatment led to a significant decrease of Sp1 and Sp3 recruitment to the ER α promoter (Fig. 5E).

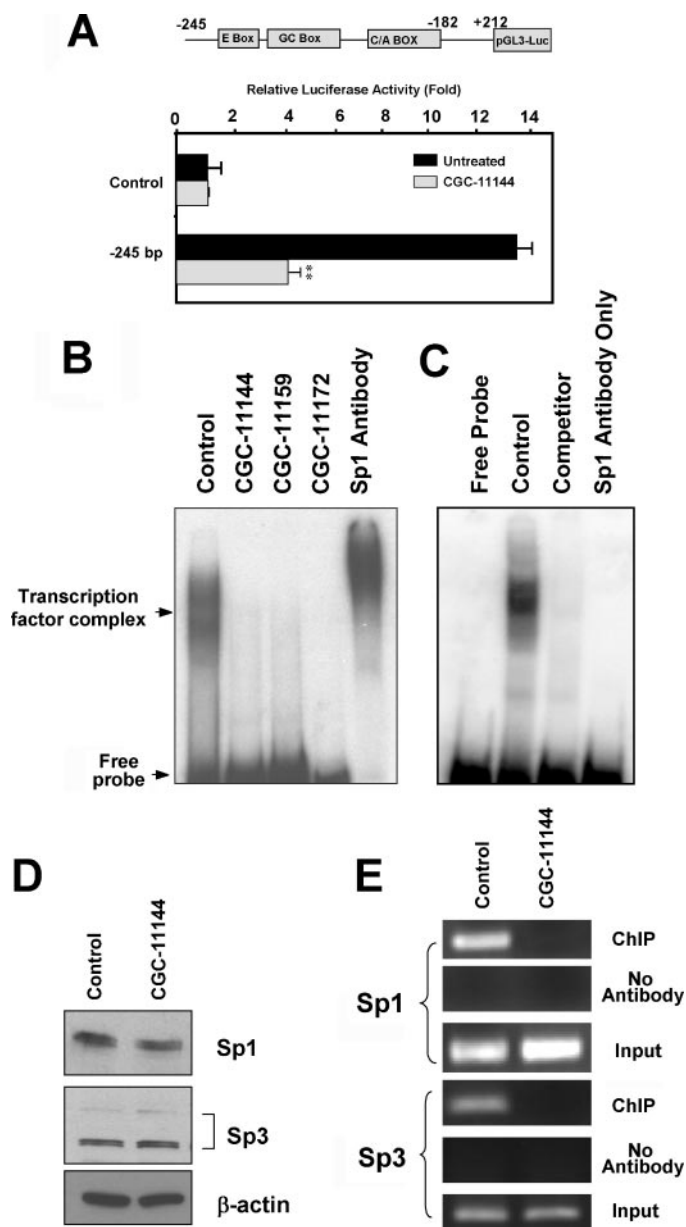


FIGURE 5. Effects of oligoamines on ER α proximal promoter activity. A, a luciferase reporter construct containing the ER α promoter fragment (-245 to $+212$ bp) was transfected into T47D cells with CMV- β -galactosidase vectors followed by treatment with $10 \mu\text{M}$ CGC-11144 for 24 h. Reporter gene activities were then measured. **, $p < 0.01$. B, T47D cells were treated with $10 \mu\text{M}$ oligoamine for 24 h. EMSA was performed using $10 \mu\text{g}$ of T47D nuclear extracts incubated with γ - ^{32}P -labeled oligonucleotides that spanned the ER α promoter from -245 to -182 bp. Super gel shift was performed by using anti-Sp1 antibody. C, EMSA was performed using γ - ^{32}P -labeled oligonucleotides of the ER α minimal promoter (-245 to -182 bp) with control T47D nuclear extracts with or without 100-fold excess of nonlabeled Sp1 consensus DNA as competitor and the Sp1 antibodies only. D, T47D cells were treated with $10 \mu\text{M}$ CGC-11144 for 24 h. Western blots were performed to detect the expression of Sp1 and Sp3. E, cross-linked chromatin prepared from untreated and CGC-11144-treated T47D cells was immunoprecipitated with Sp1 or Sp3 antibody. The immunoprecipitates were subjected to PCR analysis using primer pairs spanning the ER α promoter from -245 to -182 bp. Aliquots of chromatin taken before immunoprecipitation were used as Input controls, whereas chromatin eluted from immunoprecipitations lacking antibody were used as No Antibody controls.

Effects of JNK/AP-1 on Oligoamine-regulated ER α Expression—The cross-talk between the estrogen signaling and mitogen-activated protein kinase pathways has been intensively

studied. To date, one enhancer region termed ER-EH0 (–3778 to –3744 bp; Fig. 3B) containing an AP-1-binding site has been identified as a potential regulatory region in the ER α promoter (21). Our previous study showed that activation of the JNK/AP-1 signaling cascade protects breast cancer cells from oligoamine-induced cell death in breast cancer cells (11). To further investigate whether the JNK/AP-1 pathway is involved in regulation of oligoamine-induced ER α down-regulation, an *in vitro* JNK kinase assay was used to examine whether oligoamines affect JNK kinase activity in MCF-7 cells. c-Jun fusion protein containing the JNK phosphorylation sites was used as substrate for the kinase assay, and the JNK complex was immunoprecipitated from MCF-7 cells by using a JNK1 monoclonal antibody. The results indicate that the phosphorylation of substrate c-Jun protein (p-c-Jun) is stimulated by CGC-11144. However, simultaneous treatment with SP600125, a selective JNK inhibitor, significantly blocked both the basal JNK and CGC-11144-induced activity (Fig. 6A). SP600125 alone inhibited ER α expression, but combined treatment with SP600125 and CGC-11144 reduced ER α expression below detectable levels (Fig. 6A). These results indicate that JNK/AP-1 is a positive regulator of ER α expression and that oligoamine enhanced JNK/AP-1 activity may antagonize the down-regulation of ER α by oligoamines.

To define more precisely the effect of the JNK/AP-1 pathway on oligoamine-induced ER α down-regulation, a previously described vector expressing the c-Jun dominant negative mutant, TAM67, was stably transfected into MCF-7 cells (11). As shown in Fig. 6B, MCF-7 cells transfected with the pcDNA3.1-TAM67 vector expressed a ~29-kDa dominant negative mutant c-Jun protein. Overexpression of TAM67 inhibited ER α expression, and treatment with CGC-11144 further reduced ER α expression in TAM67-transfected cells. These results provide further evidence that the JNK/AP-1 pathway is important for ER α expression and that inhibition of this pathway may enhance down-regulation of ER α expression by oligoamines.

To further confirm that the regulation of ER α by c-Jun occurs through an effect on the promoter activity of the ER α gene, we investigated ER α protein expression in CGC-11144- and/or SP600125-treated MDA-MB-231-ER19 cells stably expressing exogenous ER α . Treatment with either SP600125 or CGC-11144 or the combination of the two agents did not alter ER α protein expression driven by the CMV promoter (Fig. 6C). This result clearly indicates that the effect of AP-1 on ER α expression occurs through transcriptional regulation on the natural promoter of ER α rather than through an effect on ER α protein itself.

DISCUSSION

Natural polyamine levels in breast tumors are usually higher than in adjacent normal tissues, and dysregulated polyamine metabolism has been well demonstrated in breast cancer (1). Our previous studies demonstrated that a novel class of polyamine analogues, oligoamines, effectively inhibits growth of human breast cancer cells in culture and nude mouse xenografts (10). However, the molecular events underlying oligoamine cytotoxicity in breast cancer cells are currently unknown. A number of recent studies have identified a wide

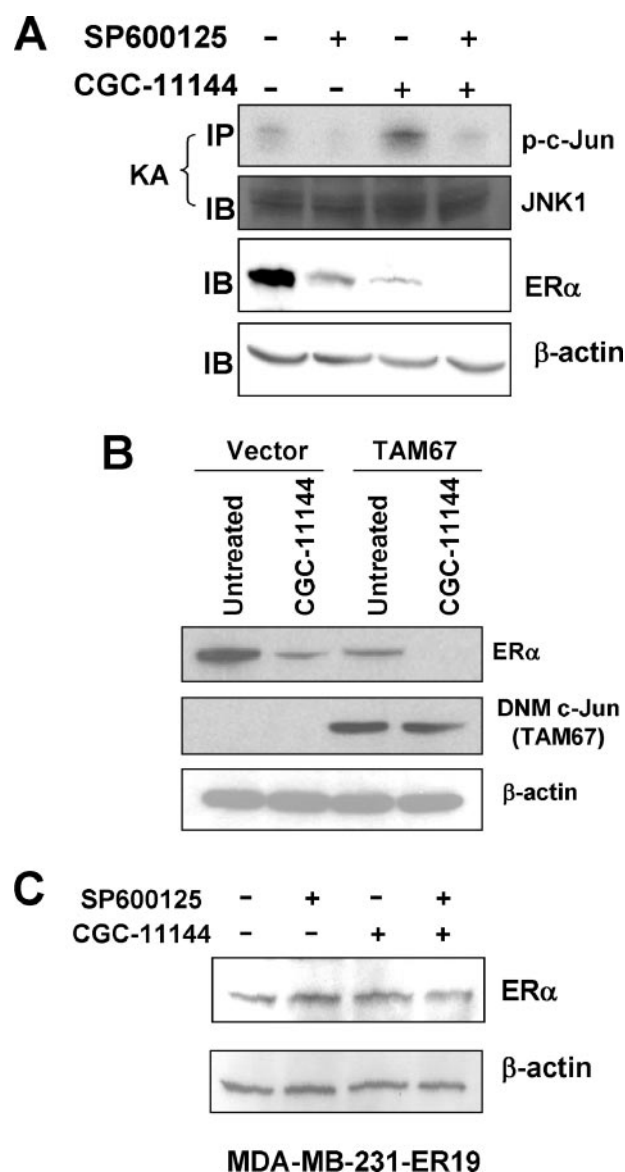


FIGURE 6. Effect of JNK/c-Jun activation on oligoamine-regulated ER α expression. A, MCF-7 cells were treated with 10 μ M CGC-11144 or SP600125 alone or both for 96 h. JNK complex was immunoprecipitated (IP) with an anti-JNK1 antibody and then was subjected to the *in vitro* kinase assay (KA) by using c-Jun fusion protein as the substrate. Total JNK protein was analyzed by Western blotting (IB) for anti-JNK1 protein as a control. Immunoblotting with anti-ER α was performed with actin protein as a control. B, empty and dominant negative mutant (DNM) c-Jun (TAM67) transfected MCF-7 cells were treated with 10 μ M CGC-11144 for 96 h, and Western blot with anti-ER α or anti-c-Jun antibodies was performed. Actin protein was blotted as a loading control. C, MDA-MB-231-ER19 cells were treated with 10 μ M CGC-11144 or SP600125 or both for 72 h. Western blots were performed by using anti-ER α and anti-actin antibodies.

range of important molecular targets for polyamine analogues in human breast cancer cells and one of these targets is ER α (1). Our current studies demonstrate that oligoamines specifically inhibit ER α protein expression but do not affect the expression of ER β or other specific steroid hormonal receptors. Oligoamine inhibition of ER α expression also leads to the down-regulation of ER α -mediated transcriptional activity and the loss of expression of several important ER α target or partner genes, including PR and cyclin D1. Based on these observations, we envision that ER α is an important molecular target of oligoam-

ines in breast cancer cells and that down-regulation of ER α may contribute to oligoamine cytotoxicity in ER-positive human breast cancer cells. There is a significant difference in the timing of the decrease in ER α expression between T-47D and MCF-7 cells by oligoamines. The possible mechanisms include variable polyamine transport, intracellular polyamine metabolism regulation, drug resistance mechanisms, etc., which may affect the accumulation of polyamines and/or analogues in tumor cells. In addition, it will be important to further elucidate whether the regulation of oligoamines on ER α target genes occurs through down-regulation of ER α or a more global effect of the analogues.

Our analyses using a series of ER α transcription activity assays demonstrate that oligoamine-inhibited ER α expression results from the suppression of ER α transcription rather than changes in protein stability. Furthermore, oligoamine treatment affects ER α transcription apparently by altering the binding of Sp1 transcription factor family members to the -245 to -182 bp proximal promoter element, which contains the GC-rich box of binding sites. It has been known for some time that polyamine-mediated conformational changes in DNA can alter the transcriptional activity of genes. Although it is still unclear whether polyamines interact with DNA in a sequence-specific manner, several studies have suggested that the GC-rich region in the major groove of DNA is one of the preferred sites for spermine binding (22–24). Polyamine-modulated protein-DNA interactions may also regulate the binding activity of regulatory proteins to specific DNA sequences in the gene promoter (25). The Sp1 transcription factor family is an important class of transcription factors whose activity may be regulated by natural polyamines. For example, a recent study found that the binding of Sp1 to the GC box of the spermidine/spermine N¹-acetyltransferase promoter is essential for transcription of this key polyamine catabolism enzyme, and a polyamine-responsive element seemed to be responsible for the elevated transcription (26). Because of structural similarity, oligoamines may strongly compete with the binding of natural polyamines to specific DNA sequences like the GC-rich regions, thus leading to the interruption of normal transcription machinery. Our recent studies indicate that co-treatment with spermine prevents the decreased expression of ER α by oligoamines, and luciferase activity assay shows that oligoamine-suppressed ERE reporter gene expression was prevented by simultaneous treatment with spermine (data not shown). However, there are multiple mechanisms by which these findings can result. Because spermine competes with the oligoamines for uptake, one likely interpretation is that spermine prevents down-regulation of ER α expression by competing with oligoamines for transport into the cells. The net effect is that the combination treatment leads to maintenance of the natural polyamine level that prevents the oligoamine-induced ER α down-regulation.

The JNK/AP-1 signaling pathway is a key component of many signal transduction pathways and plays a critical role in the control of growth in breast cancer cells. A 35-bp element called ER-EH0 mapped from -3778 to -3744 bp upstream of the ER α mRNA start site forms multiple DNA-protein com-

plexes, including an AP-1-containing complex with strong promoter activity in ER-positive breast cancer cells (21). Our data show that oligoamines activate the JNK/AP-1 signaling pathway. Use of the JNK-specific inhibitor (SP600125) or a dominant negative mutant c-Jun (TAM67) markedly inhibits ER α expression and potentiates oligoamine down-regulation of ER α expression. These findings provide evidence that activation of JNK signaling stabilizes ER α transcription and may play a role in modulating oligoamine down-regulation of ER α expression. Further elucidation of the mechanisms of the effect of the JNK/AP-1 pathway on oligoamine down-regulation of ER α gene expression may be helpful in designing treatment strategies combining oligoamines and JNK/AP-1 inhibitors.

In summary, we have demonstrated that oligoamines specifically suppress the expression and activity of ER α , a principal determinant of growth and differentiation in human breast cancer cells. Several lines of evidence suggest that oligoamines may interact with other critical regulatory proteins like Sp1 and AP-1 to mediate ER α expression. These data indicate a new approach to modulating estrogen signaling utilizing these novel analogues. These findings also suggest a relationship between polyamines and ER α expression in breast cancer cells and underscore the rationale of targeting polyamine metabolism as a potential approach to breast cancer therapy and/or prevention.

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